SOME EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE CARBOHYDRATE METABOLISM OF ETIOLATED CORN SEEDLINGS

By

CLANTON C. BLACK, JR.

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

August, 1960

ACKNOWLEDGEMENT

This writer wishes to express sincere appreciation to Dr. E. G. Rodgers for his untiring help, constant consideration, inspiring leadership and thoughtful guidance throughout the writer's entire graduate study; to Dr. T. E. Humphreys for the generous use of his laboratory facilities, patient and thorough instructions on unfamiliar techniques, stimulating discussions and thoughtful guidance in the course of this research; to Drs. O. C. Ruelke, D. O. Spinks and T. W. Stearns, for service on the committee; and to the Department of Agronomy for financial support during this graduate study.

The fullest credit is due my wife, who encouraged the writer to undertake this study, and who worked so untiringly in a determined effort to complete this step in our lives.

DEDICATION

to

my wife

Betty Louise Black

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

STATEMENT OF PROBLEM

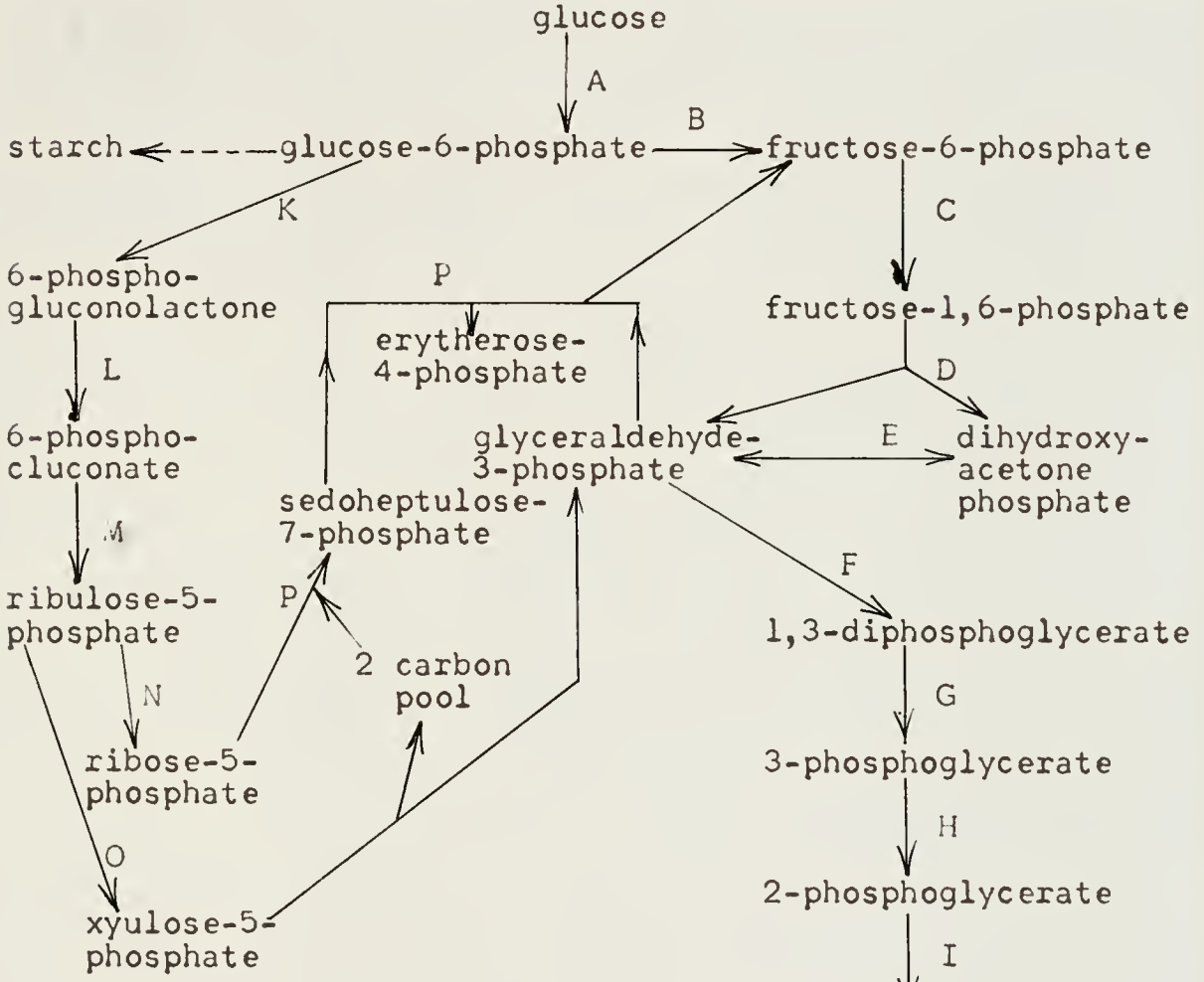
Copious amounts of research have been directed at attempting to elucidate the basic mechanism or mechanisms of action of plant growth-regulating materials. Despite the intensive and persistent efforts of research workers to solve this problem, the basic mechanism or mechanisms of action of plant growth-regulators are not known (56).

Since 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the earliest known synthetic plant growth-regulating compounds, more research has been directed at attempting to elucidate its basic mechanism of action than for any other compound. A major area of research on the basic mechanism of action of 2,4-D is its effects on plant metabolism. Recently, Humphreys and Dugger (80, 81, 82) noted that 2,4-D affected the catabolism of glucose in etiolated corn seedlings (*Zea mays* L.) by increasing the participation of the pentose phosphate pathway in glucose catabolism (Figure 1). Their *in vivo* work indicated that glucose catabolism was accommodated almost entirely via the pentose phosphate pathway in the roots of 2,4-D treated corn seedlings. These results stimulated the research in this dissertation, which was undertaken to evaluate the effect

or effects of 2,4-D on the *in vitro* activity of enzymes extracted from the roots of 2,4-D treated corn seedlings.

The basic hypothesis of this study was that 2,4-D affected the activity of an enzyme or enzymes of either the glycolytic pathway or the pentose phosphate pathway (Figure 1) or both, which results in a shift of the major pathway of glucose catabolism. To test this hypothesis, the activities of the enzymes of the control, i.e. buffer treated etiolated corn seedlings, were compared with the activities of the enzymes of the 2,4-D treated etiolated corn seedlings. This comparison was made in each experiment in this study.

The studies on the pentose phosphate pathway (Figure 1) consisted of assaying the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and studying the disappearance of ribose-5-phosphate (R-5-P) and the appearance of heptulose and hexose. The glycolytic pathway was studied by assaying for the activities of each enzyme of the pathway from glucose-6-phosphate (G-6-P) to pyruvate (Figure 1).



Glycolytic enzymes:

A. hexokinase
B. phosphoglucoisomerase
C. 6-phosphofructokinase
D. aldolase
E. triose isomerase
F. glyceraldehyde-3-phosphate dehydrogenase
G. phosphoglyceric kinase
H. phosphoglyceric mutase
I. enolase
J. pyruvic kinase

Pentose phosphate pathway enzymes:

K. glucose-6-phosphate dehydrogenase
L. gluconolactonase
M. 6-phosphogluconate dehydrogenase
N. phosphoriboisomerase
O. phosphoketopentoepimerase
P. transketolase

Figure 1. THE CATABOLISM OF GLUCOSE.

REVIEW OF LITERATURE

A prodigious amount of research was stimulated when the hormone types of plant growth-regulators such as 2,4-D were introduced in the early nineteen-forties. This growth-regulator was extensively studied, primarily because it selectively controlled dicotyledonous plants and because it was cheap, safe and easy to use. By virtue of its selectivity, the commercial use of 2,4-D was quickly developed. Hundreds of compounds have been synthesized since the introduction of 2,4-D which exhibit varying degrees of phytotoxicity. But it soon became evident and is more evident today that very little basis existed for the synthesis of new compounds and that practically nothing was known concerning the basic mechanisms whereby these compounds influenced plant growth. While it is true that many plant growth-regulating substances have been synthesized and developed to such a degree that they can be used commercially without a knowledge of their mode of action in plants, it is perhaps equally true that a clearer understanding of the modes of actions of the known growth-regulating substances could assist in making better use of the known substances and could orient the synthesis of new substances.

A complete review of the literature covering hundreds of experiments on the effects of 2,4-D on plants demonstrated several prominent factors. First, a large percentage of the data available was on the practical uses of 2,4-D in agriculture. Second, many of the studies directed at determining the effects of 2,4-D on plants were concerned with morphological or anatomical effects produced by 2,4-D at varying periods up to a year after application. Studies of this type seem to be concerned with the result of 2,4-D's action, but do not seem to be directly related to its initial mechanism of action. Third, some short-time studies, i.e. some as short as five minutes, showed that 2,4-D has some effects on plants within a fairly short period of time. Several workers have advanced the idea that to learn the basic mechanism of 2,4-D's action, studies should begin soon after its application to the plant. This approach seems logical. Therefore, this review of literature will not cite many research papers in which the data were collected long, i.e. several weeks or months, after application of 2,4-D. Fourth, although numerous hypotheses have been advanced concerning the mode of action of 2,4-D, none have completely withstood the intensive scrutiny of repeated and extended research. Fifth, 2,4-D may have several modes of action, and a search for the mode of action might be futile. Sixth, several theories as to the mode of action of 2,4-D were

reasonably supported by research data and should be considered in a study of the possible mode or modes of action of 2,4-D.

After considering the factors cited above, it was decided to limit this review of literature to research papers which deal with the probable initial mode or modes of action of 2,4-D and several physiological responses to 2,4-D.

Effects of 2,4-D on Gas Exchange

Although the effects of 2,4-D on gas exchange by numerous plants or tissues thereof have been studied extensively, these studies have not been effective in determining the mode of action of 2,4-D. Several reasons exist for this lack of effectiveness. When it is reported that 2,4-D increases or decreases or does not affect the oxygen uptake of a particular plant, this is just a general observation because many areas or sites in plant's metabolism could be stimulated or inhibited by 2,4-D. Thus, the general effect a worker might record, such as oxygen uptake or carbon dioxide (CO_2) evolution, could be caused by many reactions within a plant. Numerous other variables which could cause changes in gas exchange are the age and type of plant tissues used, concentration of 2,4-D, length of time tissue is treated, length of time between treatment and recording of experimental data, pH of the 2,4-D solution, portion of plant studied, soil moisture supply, and

and prior nutrition of the plant tissue. Because the gas exchange by 2,4-D treated tissue is dependent upon such a large number of variables, it is not surprising that increases (6,16,19,20,34,51,71,78,80,92,116,123,143) and decreases (77,110,112,147,149,157,162) or no effects (88,89,114,159) have been observed by many research workers. In experiments on the effect of 2,4-D on photosynthesis, similar types of results have been reported (36,41,47,100,114). Therefore, although the information from gas exchange studies has been useful in delineating areas for further study, it has not provided the answer to the mechanism of action of 2,4-D.

Effects of 2,4-D on Mineral Metabolism

Similarly, the same variables which influence the gas exchange from plants treated with 2,4-D also can influence other aspects of metabolism.

Wolf et al. (165) observed, 14 days after treating soybeans (Glycine max L.) growing at three levels of nitrogen (N) with a solution of 20 ppm of 2,4-D, that the potassium in treated leaves was lower, but that the potassium level in the entire plant was not affected. Similar effects were noted when tomato plants (lycopersicon esculentum Mill) (126,127) were treated with 2-methyl-4-chlorophenoxyacetic acid (MCPA) and tobacco (Nicotiana tabacum L.) with 2,4-D (164). Cooke (33) stated that within the first 24 hours after treating beans

(*Phaseolus vulgaris* L.) with 2,4-D, the uptake of potassium was markedly increased, but that after 24 hours this uptake was markedly inhibited. He postulated that a stimulation followed by an inhibition could be due to a similar effect on respiration which might control the uptake of minerals from the soil. Bass et al. (9) applied 2,4-D to a primary leaf of cranberry beans and in six days noted a higher potassium content in leaves and roots, and a lower content in stems of treated plants than in control plants. An interesting relation of potassium nutrition to translocation of 2,4-D was reported by Rice and Rohrbaugh (128). They noted that low potassium levels in tomato plants inhibited 2,4-D translocation, while increasing the potassium levels increased translocation.

Treatment with 2,4-D has been observed to inhibit the accumulation of potassium nitrate (KNO_3) in excised wheat roots (*Triticum* spp) (114), and to promote the accumulation of the salt in sugar beet leaves (*Beta vulgaris* L.) (145). Hanson and Bonner (67) stated that 2,4-D has no direct effect on the process of salt uptake, but that it has three indirect effects: 2,4-D promotes uptake of rubidium (Rb), which can be related to increased tissue hydration; pre-treatment with 2,4-D results in reduced salt uptake which is attributed to a competition between salt and water for a common energy supply; and pre-treatment with 2,4-D results in an increased capacity to gain Rb in the initial hour of absorption. This is interpreted as signifying an

increased cation exchange capacity of the tissue resulting from increased growth. Wolf et al. (165) grew soybeans at three nitrogen levels. When he treated them at the same rate, the plants at the highest level were easiest to kill, and the ease of kill was correlated with the nitrogen level. This indicates that plants in a high metabolic state were more adversely affected by 2,4-D. The data of Asana et al. (4) indicate that per unit volume, the rate of uptake of nitrogen is not affected by 2,4-D, but that the total nitrogen uptake may be reduced due to restricted root growth. Klingman and Ahlgren and Rhodes (126) reported some results which generally agree with this. Berg and McElroy (12) and Frank and Grigsby (46) reported 2,4-D treatment caused an increased nitrate content of certain weeds and crops, but in other weeds and crops the treatment had no effect.

Rakitin and Zemskaia (121) reported results of treating the susceptible bean plant in which the nitrogen uptake is sharply decreased, while in the more resistant oat (Avena sativa L.) the nitrogen uptake is only slightly decreased. He postulated that this differential response is an indication of resistance against 2,4-D by oats, and that cereals are more capable of detoxication of 2,4-D.

The metabolism of phosphorus in 2,4-D treated plants has been studied extensively due to the key role of phosphorus in plant metabolism (107,108). The results of these studies are quite variable. This variation of results

probably is caused by the same factors which were listed as influencing gas exchange measurements. Increased phosphorus uptake was observed (160) and increased inorganic phosphorus content of the entire plant (101), stems (125) and roots (9) has been reported. Decreased inorganic phosphorus content has been reported in whole plants (126,118) and in the leaves (43,120,125,164) while others have reported that 2,4-D treatment had no effect on the phosphorus content of the entire plant (165), leaves (9), stems (9,43) or roots (43,125). Rohrbaugh and Rice (130) and Fang and Butts (43) presented data which indicate that 2,4-D is not readily translocated in phosphorus deficient plants and that when phosphorus is supplied, the distribution of 2,4-D and phosphorus follows the same pattern.

Despite the contradiction in the results given above, several papers present results which indicate that a mechanism of action of 2,4-D is related to phosphorus metabolism. Fang and Butts (43) demonstrated that the incorporation of p^{32} into glucose-1-phosphate and hexosediphosphate was influenced by 2,4-D treatment. Thus, 2,4-D could be affecting phosphorus metabolism or sugar metabolism, or both. Ormrod and Williams (118) demonstrated a striking decrease in the inorganic phosphate content of soybeans within less than five minutes. Concurrently, the soluble organic phosphorus increased in the same striking manner. This work, in particular, illustrates the need for short time-intervals between treatment and analysis of the

plant to determine the initial mechanism of action of 2,4-D.

Cooke (33) noted that the uptake of calcium was enhanced initially by 2,4-D treatment, but that within 48 hours the uptake was decreased. Calcium has been reported to be higher in leaves (165) and roots (164) of 2,4-D treated plants, although Bass et al. (9) stated that six days after treatment the calcium content of all tissues of cranberry beans is lower than that of the controls.

The effect of calcium on cell wall elasticity and plasticity is of particular interest due to the demonstration by several research groups that auxin may affect the activity of pectin methylesterase (PME) (1,21,60,61,62). The general theory of the effect of calcium on cell walls is that the divalent cation reduces wall plasticity by cross-linking two carboxyl groups (1,48). Methylation of pectin is thought to increase wall plasticity by reducing the number of carboxyl groups which may be cross-linked by divalent cations (1). Ordin et al. (117) presented data which indicate that indole acetic acid (IAA) increased the formation of methylesters of pectin. These data support the hypothesis that esterification of carboxyl groups of pectin is involved in the mechanism of cell expansion. Bryan and Newcomb (21) noted that IAA stimulated the activity of PME above the control level. Glaszious and Inglis (60,61,62) presented data which indicate that auxins are effective in binding PME to cell wall preparations.

PME theoretically controls the methyl content of pectin by demethylation; thus, if auxin reduced its activity, an increase in the total methyl content of pectin could occur. Therefore, in the presence of auxin, the methyl content of pectin is increased, and consequently cell expansion is increased. If this reaction of auxin is a binding of PME, it should be insensitive to metabolic conditions such as temperature and presence of oxygen. The work of Adamson and Adamson (1) supports this idea and further substantiates the theory that auxin-induced cell wall expansion could be caused by an auxin-induced absorption of PME.

In 1955, Bennet-Clark (11) noted that the chelating substance ethylenediaminetetraacetic acid (EDTA) would act as a growth substance by stimulating the extension growth of Avena coleoptiles. Working independently, Johnson and Colmer (83,84) and Heath and Clark (68,69) reported that plant growth substances could act as chelating agents. They proposed that the growth promoting action of plant growth substances is through the binding of ions such as copper, magnesium (Mg) and calcium. Further research has generally given support to this theory (3,23,32,70,85,86), although Fawcett (44) repeated the work on changes in optical density (O.D.) due to chelation, and concluded that changes in O. D. were not due entirely to chelation. The general status of this theory today is that chelation could affect certain enzymatic reactions

and shift metabolic patterns, thus affecting growth; but this has not been substantiated and, therefore, does not eliminate the possibility that the growth-regulating molecule could have other properties which affect growth.

The effects of 2,4-D on other nutritional elements have been studied, but no conclusive results have been reported (9,33,160,165).

Effects of 2,4-D on the Metabolism of Carbon Compounds and Related Enzyme Systems

The possibility that auxins affect the metabolism of carbon compounds and play a role in enzyme activity was realized in the early research on the mechanism of action of auxin. In work with Avena coleoptile sections, Berger and Avery (13,14) noted that the activities of glutamic isocitric, alcohol and malic dehydrogenases were inhibited, enhanced, or not affected, depending upon the concentration of IAA present. Thus, they postulated a role of enzyme activator for auxins. Many research workers presently are continuing work on this basic idea, although they do not agree as to what area of metabolism is affected. Two major areas of research on the mode of auxin action have developed, one in favor of some phase of intermediary metabolism and the other in favor of changes in cell structure and the ensuing water absorption (22). It seems logical to hold to the idea that some change in the plant's metabolism could occur initially, and this could

influence changes in cell structure and subsequent water absorption.

The metabolism of nitrogen containing substances in plants as affected by 2,4-D treatment has been studied extensively. Although the results are not specific, several general effects of 2,4-D treatment have been observed which are reproducible. The total protein content of treated tissue generally is increased (34,40,49,54,124,132,133,163, 172), but decreases have been noted in leaves (34,49,50), while simultaneous protein increase in stems and roots of the same plants resulted in a total increase in protein. Galston and Kaur (55) fed labeled 2,4-D to etiolated pea stem (Pisum sativum L.) sections for 18 hours. Centrifuged fractionation revealed that the labeled fraction was localized in the centrifugal supernatant fraction devoid of all particles. When this fraction was heated, the protein from treated cells did not coagulate after ten minutes of boiling, while the untreated proteins produced a copius white precipitate under the same condition. This effect on proteins correlated closely with the effect on growth at various concentrations of 2,4-D. The treatment did not affect the total protein content. Auxin analogs which did not promote growth were less effective or completely ineffective in preventing coagulation. The effect was greatly reduced or not produced at all when growth substances were added *in vitro*. The auxin-induced alteration of the physical state of cellular

proteins may be important in explaining auxin action. Gordon (63) reported some results which indicated IAA may be associated with proteins as an absorbed, unstably bound electrolyte.

Studies on the effect of 2,4-D on amino acids have been inconclusive, with increases (2,54,99,119,124) and decreases (54,99,119) in free amino acids having been reported. A partial explanation of this type of results might be a stimulation of deaminating enzymes as reported by Moewus (113). Akers and Fang (2) exposed 2,4-D treated beans to $C^{14}O_2$ and found a large increase in the incorporation of C^{14} into aspartic and glutamic acids. They also noted a decrease in photosynthesis. Thus, they postulated more CO_2 enters through the Krebs cycle with a subsequent increase in amino acids. Boroughs and Bonner (17) found that IAA did not affect the incorporation of labeled glycine or leucine into proteins of corn and Avena coleoptiles. Luecke et al. (102) reported that the contents of thamine, riboflavin and nicotinic acids were decreased in leaves and increased in stems of 2,4-D treated beans. The activity of proteolytic enzymes in 2,4-D treated tissues has been studied with increases, decreases, or no effects on their activity being reported (48,49,50,121,124).

The effect of auxins on nucleic acid metabolism appears to be a particularly fruitful area of research. It is generally agreed among physiologists and biochemists

that nucleic acids are key components in the control of growth. Skoog (139) presented a good discussion of the role of nucleic acids in growth and presented a hypothesis linking IAA action with nucleic acid metabolism. He gave experimental results in which both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are increased in tobacco pith tissue treated with IAA (136). Croker (35) studied the effect of 2,4-D on mitosis in Allium cepa L. He noted that 2,4-D affected the nucleic acid cycle in much the same manner as ionizing radiation. Skoog (139) also noted the striking similarities in effects produced by chemical growth-regulators and ionizing radiation. As further evidence of their participation in growth, Rasch et al. (122) noted that both RNA and DNA levels increased in tumor cells from plant tissue. Rebstock et al. (125) reported that the nucleic acid phosphorus content in stem tissue from 2,4-D treated plants was approximately double that of the non-treated stem tissue. West et al. (163) noted that herbicidal concentrations of 2,4-D increased RNA content of soybean stem tissue. Other workers have noted that 2,4-D treatment increased the soluble organic nitrogen fraction in plant tissues (49,50,166).

Biswas and Sen (15) floated coleoptile sections in substrates labeled with P^{32} or C^{14} with and without IAA in a study of the incorporation of labeled compounds. After two hours the nucleic acid fraction of the sections was isolated and the radioactivity determined. The activity

was taken as an indication of the effect of IAA on incorporation and thus the effect of IAA on the cellular metabolism. DNA and RNA fractions from IAA treated tissue incubated with P^{32} counted higher, indicating that IAA affected the synthesis of nucleotides composing the nucleic acids. When incubated with labeled acetate, formate or glycine, IAA treatment did not affect the counts. He concluded that IAA stimulates the synthesis of nucleotides and phosphorylation reactions as evidenced by the tracer studies; while the compounds labeled with C^{14}, which were all known to contribute to the synthesis of purine and pyrimidine bases of nucleic acids, were not stimulated. Thus, the reactions leading to synthesis of purine and/or pyrimidine or to sugar moieties of the nucleic acids were not affected, even though the phosphorylation reactions were affected.

Key and Hanson (91), in a study of the soluble nucleotides of etiolated soybean seedlings, noted that 2,4-D induced a large increase in one fraction eluted from an ion-exchange column. When this compound was added to excised root tips or isolated mitochondria, it acted as an uncoupler of oxidative phosphorylation in a manner similar to that of dinitrophenol (DNP). Other workers have shown that 2,4-D can act as an uncoupling agent of oxidative phosphorylation (19,147). Marre and Forti (104) states that the primary effect of auxin does not depend upon phosphate acceptor availability, but more probably

involves the activation of oxidative enzymes. French and Beevers (51) agree somewhat with this view in their idea that anabolic reactions catalyzed by 2,4-D stimulate the use of adenosine triphosphate (ATP) and thus respiration. In further studies, Key et al. (92) found mitochondria from 2,4-D treated tissue to be larger and to have an increase in phosphorylative and oxidative rates when compared with mitochondria from untreated tissue. He also noted that during growth, these mitochondria increased in acid-soluble nucleotides, phospholipides, and possibly RNA. He concluded that growth induced by auxins involves a growth of mitochondria and that this growth is regulated through nucleotide metabolism.

Very little work has been done on the effect of 2,4-D on lipid metabolism. This lack of research probably is due to the lack of information on the normal metabolism of lipids. Key et al. (92) reported that 2,4-D increased the phospholipides in mitochondria during growth. Weller et al. (161) reported that the percentage of fatty acids in bean plants treated with one drop of 0.1 percent 2,4-D was not affected six days after treatment. The percentage of total lipids (ether extract) was reported by Sell et al. (131) to be slightly increased in cranberry beans following treatment with ortho, meta and para-chlorophenoxyacetic acids. The activity of castor bean (Ricinus communis Linn.) was inhibited as much as 70 percent by 2,4-D (66). Kvamme

et al. (97) reported that wheat germ and castor bean lipases were inhibited by 2,4-D with castor bean lipase being inhibited on the order of 400 times more than wheat germ lipase.

Total starch content in 2,4-D treated tissues usually is decreased (111,123,132,142,143,151,165,166,167) as are other polysaccharides (94,132). Only two papers (80,161) reported that 2,4-D had no effect on the starch or polysaccharide content. Wort and Cowie (168) reported the activity of amylase was increased in 2,4-D treated tissues, although other workers have reported that starch hydrolysis was inhibited *in vitro* by 2,4-D (18,151). Neely et al. (115) reported that the activity of both alpha and beta amylase was inhibited by 2,4-D. Salivary amylase was also reported to be inhibited *in vitro* by 2,4-D (156), but crystalline human amylase was reported to be non-responsive to 2,4-D *in vitro* (45). Most of the work with polysaccharides indicates that 2,4-D treatment increases their utilization *in vivo*. It was proposed fairly early in the search for the mode of action of 2,4-D, that carbohydrates were depleted and the plant subsequently died (111). Klingman and Ahlgren (94) stated that at death the carbohydrates would be nearly exhausted, but further research has not substantiated this theory (123,143).

The changes in sugar content of 2,4-D treated plant tissues have been reported by numerous workers. Mitchell and Brown (111) reported that sugars in 2,4-D treated morning-glory (*Ipomoea* spp) plants increased above the

controls at first, but then decreased and were nearly depleted by the third week following treatment. Using dandelions (Taraxacum officinale W.), Rasmussen (123) found that 2,4-D treatment caused an initial rapid increase in reducing sugar content of roots, but that later the reducing sugar content fell toward the level of the controls. He concluded that the action of 2,4-D on dandelion was principally one of destruction of carbohydrate reserves. Smith (142) noted that the amount of soluble sugars rose slightly by the third day following 2.4-D treatment, then fell steadily. Buckwheat plants (Fagopyrum esculentum M.) sprayed with 2,4-D were analyzed for total sugars by Wort (167). He found total sugar increased in the stem the first two days but fell below the controls afterward; total sugar in roots and leaves declined steadily. Similar results also were reported later (166).

Both reducing and non-reducing sugars were lower in stems of 2,4-D treated beans (132) and wild garlic (Allium vineale L.) (94). Weller et al. (161) reported that non-reducing sugars were depleted in bean leaves and roots following 2,4-D treatment. Wolf et al. (165) reported reducing sugars were consistently higher in treated plants. Two groups of workers reported that reducing sugar content was not affected by 2,4-D treatment (80,161). Skoog and Robinson (140) incubated tobacco stem segments with various concentrations

of IAA for several months. They noted that reducing sugar content increased in all cases.

In 1956, Humphreys and Dugger (78) began a series of experiments on the effects of 2,4-D on plant metabolism. They noted that, although 2,4-D treatment increased the rate of respiration of etiolated pea seedlings, the respiratory quotient of both treated and untreated seedlings remained near 1.0. These results suggested that carbohydrate was the major substrate being oxidized in both treated and untreated seedlings. In 1957, they (80) reported that the reducing sugar, sucrose and starch contents of 2,4-D treated and untreated seedlings were essentially the same, thus concluding that the higher rate of respiration in 2,4-D treated seedlings was not due to a greater amount of respiratory substrate being present in these seedlings. The pathways of glucose catabolism in both 2,4-D treated and untreated root tips of pea, corn and oat seedlings were evaluated in short-time experiments by collecting the $C^{14}O_2$ evolved when glucose-1-C^{14} and glucose-6-C^{14} were supplied as substrates (79). This work was based on the idea that if glucose were broken down via the glycolytic pathway, the rate of $C^{14}O_2$ production from the first and the sixth carbon of the glucose molecule should be the same. If, on the other hand, glucose were broken down via the pentose phosphate pathway, the rate of $C^{14}O_2$ production from the first carbon of the glucose molecule would initially be greater

than that from the sixth carbon. In these experiments, they found that 2,4-D caused an increase in the amount of glucose catabolized via the pentose phosphate pathway. They postulated that 2,4-D increases respiration by causing more glucose to be catabolized via the pentose phosphate pathway (80).

They further demonstrated by feeding labeled substrates, i.e. glucose, pyruvate, succinate and acetate, that both 2,4-D and DNP promoted catabolism of exogenous substrates by blocking synthetic metabolic pathways in intact etiolated corn root tips (81). Further evidence of glucose catabolism via the pentose phosphate pathway in 2,4-D treated tissue was obtained using labeled glucose and labeled gluconate (82). They concluded that in etiolated corn root tips the catabolism of glucose, when 10^{-3} molar (M) 2,4-D was used, was almost totally accommodated via the pentose phosphate pathway. Fang et al. (42) fed labeled glucose to bean stem tissues and concluded that 2,4-D treatment caused an increase in the amount of glucose catabolized via the glycolytic sequences. The variation in the results of Fang et al. and those of Humphreys and Dugger could be caused by several factors: Humphreys and Dugger's results were obtained immediately following 2,4-D treatment, whereas Fang et al. used tissue which had been treated seven days prior to the study; difference in plant tissues

used; and the point made by Humphreys and Dugger (82), that evaluation of catabolic pathway of glucose based only on the yield of $C^{14}O_2$ from labeled glucose is not possible. Thus, the work by Humphreys and Dugger strongly indicated that 2,4-D treatment increased glucose catabolism via the pentose phosphate pathway as opposed to the normal glycolytic scheme. These results stimulated the research of this thesis.

MATERIALS

Plant materials. Corn seed (var. Dixie 18) were soaked in distilled water 24 hours with aeration and placed in porcelain trays on moist paper towels. The trays were covered with a sheet of aluminum foil and placed in the dark at 22° C for 60 hours. The etiolated seedlings were divided into two groups and then were placed in glass microscope slide trays with the roots down and the cotyledons resting on the microscope slides. One group of the seedlings was treated by immersing the roots in phosphate buffer, pH 5.3, 10^{-2} M. The other group of seedlings was treated by immersing the roots in buffer plus 2,4-D, 10^{-3} M. The trays were placed in the dark for 12 hours at 22° C. After a 12-hour treatment period the seedlings were removed, washed, blotted dry and the roots excised. The roots from each group were weighed and used to prepare enzyme extracts.

Preparation of enzyme extracts. Two kinds of extracts were prepared from each group of roots. Cell-free extracts and acetone powder extracts were prepared from each group. The same procedure was followed for both 2,4-D and buffer treated roots.

Ten grams of excised roots were added to 100 milliters (ml) of water (4-6° C) and placed under refrigeration for about 30 minutes. The water was decanted off and the roots were placed in an ice-cold mortar containing 10.0 ml of 0.05 M tris (hydroxymethyl) aminomethane (Tris), pH 7.4 plus 1.0 ml of 1.0 M EDTA. The roots were ground until no intact roots were visible. The resulting homogenate was filtered through four layers of cheesecloth, and the resulting filtrate centrifuged at 900 X gravity (G) for ten minutes at 0° C.

The yellowish-brown supernatant fraction was decanted and its pH adjusted to 7.0 with dilute sodium hydroxide (NaOH). The supernatant (12-18 ml) then was placed in cellophane tubing and dialyzed overnight under refrigeration against two 500 ml portions of 0.01 M Tris, pH 7.4. The dialyzing solution was changed about 10:00 p.m. each evening. After dialysis, the pH of the dialyzed supernatant was adjusted to 7.4 with dilute NaOH. This dialyzed supernatant was designated as a cell-free extract. Cell-free extracts were used the same day they were prepared.

The procedure followed in preparation of the acetone powder extracts is essentially that given by Hageman and Arnon (65). Ten grams of corn roots were added to 100 ml of water (4-6° C) and placed in the refrigerator for about one hour. The water was decanted off and the roots were ground until no intact roots

were visible in an ice-cold mortar which contained 15 ml of 0.1 M phosphate buffer, pH 8.2, which was 0.03 M with respect to EDTA. Cold acetone (125 ml, -15° C) was added slowly with stirring to the homogenate. The resulting slurry was immediately filtered with suction through Whatman number one filter paper on a Buchner funnel. The precipitate was washed three times with 75 ml portions of cold acetone (-15° C) and left under suction until free of an acetone odor. The filter paper containing the precipitate was placed in a vacuum dessicator and evacuated with a water aspirator for 15 minutes. The vacuum was released and phosphorus pentoxide (P_2O_5) was added to the dessicator, a vacuum was drawn and the precipitate was dried *in vacuo* for 12 hours. Then the acetone powders were stored in a dessicator, over calcium chloride, in the refrigerator.

Acetone powder extracts were prepared by an extraction procedure which consisted of stirring the filter paper plus the acetone powder (a fibrous yellow material) for 15 minutes in 25 ml of a solution which contained 0.01 M phosphate buffer and 0.0015 M EDTA, pH 7.2. The resulting slurry was centrifuged at 10,000 X G for five minutes at -5° C, then filtered through Whatman number one filter paper. The resulting filtrate was designated as the acetone powder extract. All extracts were used the same day they were prepared.

The nitrogen content of each extract was determined by digesting 0.1 ml of the extract in 1.0 ml of sulfuric acid, followed by Nesselerization. Therefore, the activity of each enzyme in each extract is presented on a nitrogen or protein basis. The nitrogen concentration was multiplied by 6.25 to obtain protein concentration.

EXPERIMENTAL PROCEDURE

Studies on the Pentose Phosphate Pathway

Pentose disappearance. Ribose-5-phosphate was used as the substrate in these studies on pentose disappearance. Reaction mixtures were prepared, using cell-free and acetone powder extracts, which contained R-5-P. At the times zero and 60 minutes, the concentrations of pentose, heptulose and hexose were determined. To support these studies, chromatographs were made of solutions prepared from the reaction mixtures at zero and 60 minutes.

Each reaction mixture used for studying the disappearance of pentose and the appearance of heptulose and hexose was prepared in a long, narrow test tube in a water bath at 38°C. The reaction was started by adding the cell-free or acetone powder extract. Each reaction mixture consisted of: 0.2ml of triphosphopyridine nucleotide (TPN) (4 milligrams (mg)/ml); 0.1 ml of flavinadenine mon-nucleotide (FMN) (1 mg/ml); 0.1 ml of $MgCl_2$ (0.1 M); 3.4 ml of Tris buffer (pH 7.4, 0.1 M); 0.2 ml of R-5-P (0.1 M); and 2.0 ml of cell-free or acetone powder extract to give a total of 6.0 ml.

At the timed increments of zero and 60 minutes after starting the reaction, aliquots (1.0 or 2.0 ml) of the

reaction mixture were taken and immediately mixed with equal volumes of 10.0 percent trichloroacetic acid (TCA) to stop the reaction. The resulting mixture was centrifuged at 900 X G for five minutes to remove protein. The supernatant fraction was used to determine pentose, heptulose and hexose concentrations in the reaction mixture at the indicated times.

Pentose was determined colorimetrically by the orcinol method of Bail (8) as modified by Mejbaum (109). When reacted with orcinol, pentoses yield a product with a maximum absorption at 670 millimicrons (mu). Heptulose also was determined with orcinol following the general procedure of Horecker and Smyroniotis (74). The heptulose maximum absorption occurs at 580 mu. Horecker and Smyroniotis (74) outlined the procedure followed in the pentose and heptulose determinations. The concentrations of pentose and heptulose in an unknown solution can be calculated from density measurements at 670 mu and 580 mu, which are obtained from the unknown solution and from known standards of the pentose, arabinose, and the heptulose, sedoheptulosan.

Hexoses were determined colorimetrically by the method of Dische et al. (37). Ashwell (5) outlined the procedure followed in these experiments. To determine hexose, 1.0 ml of the supernatant fraction described above was added to 4.95 ml of a mixture of six parts of sulfuric acid to one part of water, cooled, then boiled

three minutes, cooled, 0.11 ml of cysteine hydrochloride added, mixed and allowed to stand for two hours at room temperature. The hexose concentration then was determined by obtaining the O.D. readings at 415 and 380 mu and comparing these O.D. readings with those obtained from known concentrations of the hexose, glucose.

Samples of known sugars were run in preliminary experiments to test the reliability of the procedures described above. In the range of sugar concentrations used in these experiments, the procedures proved to be quite reproducible. Horecker and Smyroniotis (74) indicated that fructose interfered with the pentose determination, but in these preliminary experiments it did not.

Chromatographic studies on pentose disappearance. These chromatographic studies were designed to support the studies on pentose disappearance and the appearance of heptulose and hexose. The same reaction mixture used in studying the disappearance of pentose was used. At the timed increments of zero and 60 minutes, 3.0 ml samples of the reaction mixture were removed and placed in a boiling water-bath for ten minutes. The samples were cooled and then incubated at 38°C for 30 minutes with 1.0 ml of alkaline phosphatase (1 mg/ml), pH 9.5, to hydrolyze the phosphate sugars. The samples again were placed in a boiling water-bath for ten minutes. The samples were

centriguged and the supernatant fraction was decanted off and evaporated with heat and vacuum to about 0.3 ml volume. The solution was mixed with two ml of absolute ethanol and evaporated to dryness. The resulting precipitate was dissolved in 0.5 ml of water and used as the sample for studying sugars chromatographically.

The filter paper (Schleicher and Schuell, number 589 blue) used in these ascending chromatographic studies was pre-washed with oxalic acid. From 10 to 50 lambda spots were made, one inch from the bottom of the paper. Large sheets of filter paper (18 X 24 inches) were used. These sheets were formed into cylinders and were equilibrated for at least six hours by suspension over the solvent, prior to placing them in the solvent. The solvent system used was n-butanol-pyridine-water in the ratio 3:2:1.5 for one-dimensional chromatographs. When two-dimensional chromatographs were run, water-saturated phenol was the second solvent. The orcinol spray of Klevstrand and Nordal (93) was used for ketoses, particularly sedoheptulose, while the B-naphtholamine and phosphoglucinol sprays given by Smith (144), were used to detect other sugars. Identification of the sugars in the ethanol solution was made by comparing their Rf values and colors with those obtained from known sugars. The known sugars were processed using the same procedures given above for the unknown sugars.

Assays for specific enzymes. The procedures described in the remainder of this dissertation will be concerned with assays for the activity of specific enzymes. In assaying for each enzyme, preliminary experiments, not reported in this dissertation, were run, in which the limiting concentration of enzyme was determined for each reaction. It was considered that the range of limiting enzyme was obtained when the amount of enzyme could be doubled to result in the amount of product being doubled in a fixed time interval.

Glucose-6-phosphate dehydrogenase. The presence of this enzyme was studied by measuring the reduction of TPN at 340 mu with a Beckman DU spectrophotometer, with G-6-P as the substrate in the following reaction:

$$\text{G-6-P} + \text{TPN}^{+} \rightleftharpoons \text{6-PHOSPHOGLUCONATE} + \text{TPNH} + \text{H}^{+}$$

The assay mixture contained the following components: 2.0 ml of Tris buffer (pH 7.4, 0.1 M); 0.1 ml of TPN (4 mg/ml); 0.1 ml of $MgCl_2$ (0.1 M); 0.1 ml of G-6-P (0.1 M); cell-free extract; and water to 3.0 ml total volume in Beckman corex cells of one centimeter (cm) light path. TPN was omitted from the blanks, and the reactions were started by adding the extract. The increase in O.D. was followed at timed intervals for periods up to 30 minutes.

6-Phosphogluconate dehydrogenase. The presence of this enzyme was studied by measuring the reduction of TPN at 340 mu, employing the same procedure as was used in assaying for G-6-P dehydrogenase. The same assay mixture was used except 0.1 ml of 6-phosphogluconate (6-PGA) (0.1 M) was used as the substrate in the reaction given below:

$$\text{6-PGA} + \text{TPN}^{+} \rightleftharpoons \text{RIBULOSE-5-PHOSPHATE} + CO_2 + \text{TPNH} + H^{+}.$$

Studies on the Glycolytic Pathway

Phosphoglucoisomerase. A colorimetric method, based on the color formed by fructose-6-phosphate (F-6-P) in the presence of resorcinol (129), was used to determine the F-6-P formed in the reaction below:

$$\text{G-6-P} \longleftrightarrow \text{F-6-P}.$$

F-6-P gives about 65 percent of the color of free fructose in this procedure (141). A typical reaction mixture consisted of: 0.2 ml of Tris buffer (pH 9.0, 0.05 M); 0.2 ml of G-6-P (0.1 M); and 0.1 ml of cell-free extract. The mixtures were incubated for ten minutes at 38°C. The reaction was stopped by adding 3.5 ml of 8.3 M HCl. One ml of 0.1 percent resorcinol in 95 percent ethanol was added and the mixture heated for ten minutes at 80° C, then cooled in a water bath at room temperature. The color intensity then was read with a Klett colorimeter using filter number 54 (500-570 mu). After determining the

limiting enzyme concentration, the reaction mixture given above was incubated for periods of 10, 15 and 20 minutes, and the micromoles (umoles) of F-6-P were determined by calculations from the Klett readings of known samples of F-6-P.

6-Phosphofructokinase. 6-phosphofructokinase, which catalyzed reaction 1 below, was measured by the procedure of Ling et al. (98), using the complete system shown in equations 1, 2 and 3 below. In this system, F-6-P and ATP are substrates, and Mg is an essential co-factor.

1. F-6-P + ATP $\longleftrightarrow$ F-1,6-P + ADP.
2. F-1,6-P $\longleftrightarrow$ DIHYDROXYACETONE PHOSPHATE + GLYCERALDEHYDE-3-PHOSPHATE.
3. DIHYDROXYACETONE PHOSPHATE + DPNH $\longleftrightarrow$ ALPHA-GLYCEROPHOSPHATE + DPN^{+}.

The fructose-1,6-phosphate (F-1,6-P) formed in reaction 1 is cleaved by aldolase, and the dihydroxyacetone phosphate which is formed is reduced through the utilization of reduced diphosphopyridine nucleotide (DPNH) to alpha-glycerophosphate. The oxidation of DPNH in reaction 3 was followed with a Beckman DU spectrophotometer at 340 mu, as a measurement of the activity of 6-phosphofructokinase. A typical reaction mixture in a Beckman corex of one cm light path consisted of: 0.5 ml of tris buffer (pH 8.0, 0.2 M); 0.3 ml of ATP (0.1 M); 0.2 ml of F-6-P (0.1 M); 0.3 ml of $MgCl_2$ (0.01 M); 0.1 ml of cysteine-hydrochloride (0.2 M); 0.1 ml of DPNH (2 mg/ml); 0.1 ml

of aldolase (1 mg/ml); 0.05 ml of alpha-glycerophosphate dehydrogenase (1 mg/ml); 0.1 ml of cell-free extract; and water to 3.0 ml total volume. The blank consisted of the complete reaction mixture as given above, minus DPNH. The reactions were started by adding F-6-P. The endogenous activity of the reaction mixture without the extracts also was determined. This activity was subtracted from the measurements obtained with the extract to give the activity of 6-phosphofructokinase in the extracts.

Aldolase. Aldolase activity was measured by the method of Sibley and Lehninger (134) as modified by Beck (10), in which the triose phosphates formed in the reaction below are trapped with hydrazine:

F-1,6-P $\rightleftharpoons$ DIHYDROXYACETONE PHOSPHATE + GLYCERALDEHYDE-3-PHOSPHATE.

The triose phosphate hydrazones formed were treated with alkali, and the color produced by the addition of 2,4-dinitrophenylhydrazine (2,4-DNPH) and NaOH was read with a Klett colorimeter using filter number 54. A typical reaction mixture consisted of: 1.0 ml of Tris buffer (pH 8.6, 0.1 M); 0.25 ml of F-1, 6-P (0.05 M); 0.25 ml of hydrazine (0.22M); 0.2 ml cell-free extract; and 0.8 ml of water. The reaction mixtures were incubated at 38°C for ten minutes with various amounts of extract. Results of the assay are presented in a graph

of ml of extract versus Klett readings per mg N to demonstrate a typical experimental result. The absolute amounts of triose phosphates were not determined, but since their concentration is proportional to the Klett readings, the Klett readings are taken as an indication of enzyme activity.

Glyceraldehyde-3-phosphate dehydrogenase. Activity of glyceraldehyde-3-phosphate dehydrogenase (GPDH) could not be demonstrated in cell-free extracts; therefore, acetone powders prepared following the basic method of Hageman and Arnon (65) were used to study the activity of this enzyme. GPDH was measured in a Beckman DU spectrophotometer by the optical test of Warburg and Christian (158) based on the increased O.D. of reduced DPN and TPN at 340 mu in the reaction below:

$$\text{GLYCERALDEHYDE-3-PHOSPHATE} + DPN^{+} + H_3PO_4 \longleftrightarrow$$
$$\text{1,3-DIPHOSPHOGLYCERATE} + DPNH + H^{+}.$$

The following reaction mixtures were prepared in Beckman corex cells of one cm light path: 0.2 ml of DPN (4 mg/ml); 0.1 ml of sodium arsenate (0.17 M); 0.1 ml of potassium flouride (0.1 M); 0.15 ml of reduced glutathione (0.1 M); 0.1 ml of glyceraldehyde-3-phosphate (G-3-P) (40 mg/6 ml); 1.5 ml of Tris buffer (pH 8.5, 0.1 M); 0.02 ml of acetone powder extract; and water to 3.0 ml total volume. Substrate was omitted in the blanks. Glutathione and G-3-P were prepared fresh daily. Reactions were started by

adding either the extract or substrate. The increase in O.D. at 340 mu was followed at timed intervals. Results are presented on the basis of the change in O.D. per mg N versus time.

Phosphoglyceric kinase. The activity of phosphoglyceric kinase was determined by the procedure of Axelrod and Banduski (7) in which the 1,3-diphosphoglycerate (1,3-DPGA) formed from 3-phosphoglyceric acid (3-PGA) in the reaction below, was trapped by hydroxylamine, forming hydroxamic acid:

$$3\text{-PGA} + \text{ATP} \longleftrightarrow 1,3\text{-DPGA} + \text{ADP}.$$

The hydroxamic acid formed a colored ferric complex when reacted with ferric chloride, which was read with a Klett colorimeter using filter number 54. A typical reaction mixture consisted of: 0.4 ml of a solution of hydroxylamine (2.5 M) plus $MgCl_2$ (0.015 M); 0.5 ml of 3-PGA (0.052 M); 0.2 ml of ATP (0.1 M); 0.15 ml of Tris buffer (pH 7.4, 0.05 M); and 0.05 ml of cell-free extract. The blank consisted of the same reaction mixture, except 3.0 ml of a ferric-chloride ($FeCl_3$) -HCL-TCA mixture was added prior to adding the extract. The reaction mixtures were incubated at 37° C in a water-bath for periods of 10, 15, 20 and 25 minutes. The reactions were stopped by adding 3.0 ml of the $FeCl_3$-HCl-TCA mixture.

Carboxylase. When the enzymes phosphoglyceric mutase, enolase and pyruvic kinase were studied, pyruvate was measured as the product. If carboxylases are present, pyruvate could undergo one or all of the reactions given below (137,155):

1. $CH_3 \cdot CO \cdot COOH \longleftrightarrow CH_3 \cdot CHO + CO_2$
2. $CH_3 \cdot CO \cdot COOH + CH_3 \cdot CHO \longleftrightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + CO_2$
3. $2\ CH_3 \cdot CHO \longleftrightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3$.

Obviously, if pyruvate reacted in this manner when pyruvate was measured, a stable product was not being measured, but rather a substrate for another reaction. Thus, if carboxylases are present in the extracts, pyruvate can not be measured to indicate the activity of phosphoglyceric mutase, enolase and pyruvic kinase. Therefore, carboxylase activity was determined manometrically by CO_2 evolution in the presence of pyruvate, as the substrate following the method of Singer and Pensky (137,138). The following components were placed in standard 15 ml conical Warburg vessels, and CO_2 was checked by the direct method as given by Umbreit et al. (152): in the main compartment, 1.0 ml of succinate buffer (pH 6.0, 0.2 M); 0.6 ml of 1,1-dimethyl-3,5-diketocyclohexane (0.05 M); 0.1 ml of thiamine pyrophosphate (ThPP) (5.8×10^{-4} M); 0.2 ml of $MgCl_2$ (0.01 M); 0.1 ml serum albumin (one percent); cell-free or acetone powder extract; water to 2.8 ml total volume; and in the side arm,

0.2 ml of sodium pyruvate (0.5 M). At zero time, the pyruvate was tipped into the main compartment and CO_2 evolution was followed for periods up to two hours.

Phosphoglyceric mutase, enolase and pyruvic kinase. This group of enzymes catalyze reactions 1, 2 and 3, respectively, below:

1. 3-PHOSPHOGLYCERATE $\leftrightarrows$ 2-PHOSPHOGLYCERATE.
2. 2-PHOSPHOGLYCERATE $\leftrightarrows$ PHOSPHO-ENOL-PYRUVATE + H_2O.
3. PHOSPHO-ENOL-PYRUVATE + ADP $\leftrightarrows$ PYRUVATE + ATP.

In assaying for these enzymes it was reasoned that each one could be studied, if the necessary co-factors and substrates were added, by assaying for pyruvate. Pyruvate was determined in these studies by the method of Friedemann and Haugen (52) as modified by Kachmar and Boyer (87).

Phosphoglyceric mutase activity was assayed using the following reaction mixture adapted from Grisolia et al. (64): 0.2 ml of 3-PGA (0.375 M); 1.5 ml of Tris buffer (pH 7.4, 0.1 M); 0.2 ml of $MgCl_2$ (0.1 M); 0.2 ml of ADP (0.1 M); 0.1 ml of 2,3-diphosphoglycerate (2,3-DPGA) (30 mg/5 ml); 0.1 ml of KCl (0.5 M); 0.2 ml of extract; and water to 2.5 ml total volume. Phosphoglyceric mutase activity was measured in both cell-free and acetone powder extracts.

Enolase activity was assayed in the following reaction mixture by utilizing the activity of a phosphatase in the extracts, which would hydrolyze phospho-enol-pyruvate (PEP)

to pyruvate: 1.5 ml of Tris buffer (pH 7.4, 0.1 M); 0.2 ml of 2-phosphoglycerate (2-PGA) (0.1 M); 0.2 ml of $MgCl_2$ (0.1 M); 0.3 ml of cell-free extract and water to 2.5 ml total volume. 2-PGA was made fresh daily. Using 0.2, 0.3 and 0.4 ml of extract to start the reactions, the reaction mixtures were incubated ten minutes at 38° C. Pyruvate was determined after the reactions were stopped with 2,4-DNPH. The results of colorimetric determinations of pyruvate are presented on the basis of Klett readings per mg N versus ml of extract added.

Pyruvic kinase activity was measured by the procedure of Kachmar and Boyer (87), in which the activity of the enzyme is based on the rate of formation of pyruvate. A complete reaction mixture had the following components: 1.5 ml of Tris buffer (pH 7.4, 0.1 M); 0.2 ml of PEP (0.05 M); 0.2 ml of $MgCl_2$ (0.1 M); 0.2 ml of ADP (0.1 M); 0.1 ml of KCl (0.5 M); 0.1 ml of extract; and water to 2.5 ml total volume. The reaction mixtures were incubated for ten minutes at 38° C. Reactions were started by adding enzyme extract and stopped by adding 2,4-DNPH. The blank consisted of a complete reaction mixture minus PEP, which was added after the 2,4-DNPH. The reaction mixtures then were assayed for pyruvate. McCollum et al. (105,106) noted that reaction mixtures minus ADP were active, indicating the possible hydrolysis of PEP by a phosphatase, as illustrated in the following reaction:

$$PEP + H_2O \longleftrightarrow PYRUVATE + INORGANIC\ PHOSPHATE.$$

Phosphatase activity was determined in both acetone powder extracts and cell-free extracts by omitting ADP from the reaction mixture given above. The endogenous phosphatase activity was subtracted to obtain the pyruvic kinase activity.

Reagents used in these studies. The chemicals used in these studies were obtained from the following sources: 2,4-D from the Eastman Kodak Company; ribose from Eastman Organic Chemicals; F-1,6-P from Mann Research Laboratories, Incorporated; F-6-P and sodium pyruvate from Nutritional Biochemicals Corporation; DPN and DPNH from Pabst Laboratories; R-5-P, G-6-P, ATP, G-3-P, 3-PGA, 2,3-DPGA and ThPP from Schwarz Bioresearch Incorporated; and TPN, TPNH, 6-PGA, F-6-P, ADP, PEP, 2-PGA and FMN from Sigma Chemical Company. Sedoheptulosan was generously donated by Dr. N. K. Richtmyer of the National Institute of Health, to whom the author is indebted. The enzymes used in these studies were obtained from the following sources: aldolase from Mann Research Laboratories, Incorporated; alkaline phosphatase from Nutritional Biochemicals Corporation; and alphaglycerophosphate dehydrogenase from Sigma Chemical Company. Bovine serum albumin was obtained from Mann Research Laboratories, Incorporated.

The 2,4-D was neutralized with NaOH and the sodium salt recrystallized twice from a water-alcohol solution. The following compounds were obtained as barium salts and converted to potassium salts by the addition of a slight

excess of potassium sulfate to a solution of the barium salts in dilute acid: G-6-P, R-5-P, F-6-P, F-1,6-P, 2-PGA, 6-PGA, 2,3-DPGA and 3-PGA. Monobarium DL-glyceraldehyde-3-phosphate diethylacetal was converted to DL-G-3-P by mixing with and aqueous suspension of Dowex 50, heating the suspension and centrifugation to remove the resin. PEP was obtained as the silver barium salt and converted to the sodium salt by tituration with a slight excess of HCl followed by the addition of a bare excess of sodium sulfate (87). The other chemicals used were the highest grades commercially available and were used without further purification.

RESULTS

Studies on the Pentose Phosphate Pathway

Pentose disappearance. The enzymes which catalyze the utilization of pentose and the formation of heptulose and hexose were active in extracts from both treated and untreated tissue. In Table 1, all experiments indicated that pentose was utilized and heptulose and hexose were formed in the 60-minute incubation period.

Experiments 1, 2, 3, 4 and 5 demonstrate the disappearance of pentose and the appearance of heptulose and hexose in cell-free extracts which were made from the roots of control and treated corn seedlings. Experiments 6 and 7 were included to demonstrate the same system in acetone powder extracts, in untreated cell-free extracts and in untreated cell-free extracts with 2,4-D added to the reaction mixture. Obviously cell-free extracts (Experiments 1, 2, 3, 4, 5 and 7) were much more active than acetone powder extracts (Experiment 6) in utilizing pentose and in forming heptulose and hexose.

In Table 1 it should be noted that the results of each experiment are presented on the basis of the number of umoles per six ml and on the basis of the number of umoles per mg N per six ml. The percent change due to 2,4-D is based on the number of umoles per mg N per six ml.

TABLE 1

PENTOSE UTILIZATION AND FORMATION OF HEPTULOSE AND HEXOSE*

Experiment Number	Treatment**	Pentose Utilized			Heptulose Formed			Hexose Formed		
		umoles/ 6 ml	umoles/mg N/ 6 ml	% Change due to 2,4-D	umoles/ 6 ml	umoles/mg N/ 6 ml	% Change due to 2,4-D	umoles/ 6 ml	umoles/mg N/ 6 ml	% Change due to 2,4-D
1	Buffer	8.7	9.3	----------	4.1	4.4	----------	1.9	2.0	----------
	2,4-D	11.5	11.1	plus 19.0	5.3	5.1	plus 16.0	3.1	3.0	plus 50.0
2	Buffer	9.1	12.3	----------	4.3	5.8	----------	2.5	3.4	----------
	2,4-D	11.4	15.8	plus 29.0	4.6	6.4	plus 10.0	2.8	3.9	plus 15.0
3	Buffer	9.2	10.4	----------	3.9	4.1	----------	1.7	1.8	----------
	2,4-D	13.4	13.7	plus 32.0	4.3	4.4	plus 7.0	3.9	4.0	plus 122.0
4	Buffer	9.7	10.3	----------	1.9	2.0	----------	2.1	2.5	----------
	2,4-D	12,4	11.1	plus 8.0	3.8	3.4	plus 70.0	4.3	3.8	plus 52.0
5	Buffer	10.6	9.2	----------	2.9	2.6	----------	2.9	2.6	----------
	2,4-D	13.4	12.0	plus 30.0	4.4	3.9	plus 50.0	3.1	2.8	plus 8.0

TABLE 1 (continued)

6	Buffer***	2.4	3.1	----------	0.0	0.0	----------	0.7	0.9	----------
	2,4-D****	4.1	5.2	plus 68.0	0.5	0.6	----------	0.9	1.2	plus 33.0
7	Untreated	9.9	15.4	----------	3.7	5.8	----------	2.2	3.4	----------
	Untreated****	9.4	14.7	minus 5.0	4.4	6.9	plus 19.0	2.3	3.5	plus 3.0

*The reaction mixture contained: TPN, 800ug; FMN, 100 ug; $MgCl_2$, 10 uM; Tris buffer, pH 7.4, 340 uM; R-5-P, 20 uM; and 2.0 ml of extract to give a total volume of 6 ml. The reaction mixtures were incubated for 60 minutes at 38°C prior to determining the concentrations of the sugars given above.

**Three-day-old etiolated corn seedlings treated 12 hours with either 10^{-2} M phosphate buffer, pH 5.3 or buffer plus 10^{-3} M 2,4-D.

***Acetone powder extracts.

****2,4-D added to the reaction mixture to make a final concentration of 3.3×10^{-5} M.

The reaction mixtures contained 20 umoles of pentose per six ml at time zero. The pentose utilized (Table 1) in the 60-minute incubation period varied from 2.4 umoles (Experiment 6) to 13.4 umoles (Experiments 3 and 5). The formation of heptulose per six ml varied from 1.9 umoles (Experiment 4) to 5.3 umoles (Experiment 1) in the cell-free extracts. Acetone powder extracts (Experiment 6) formed only a small amount of heptulose. Hexose formed per six ml with cell-free extracts varied from 1.7 umoles (Experiment 3) to 4.3 umoles (Experiment 4). Acetone powder extracts (Experiment 6) formed 0.9 umoles of hexose.

The umoles of endogenous hexose at zero time was subtracted when necessary. A correction for hexose at zero time was necessary only in four instances, because the extracts were dialyzed to remove sugars and other soluble substances. The greatest amount of hexose found at zero time was 0.08 umoles. At zero time, heptulose was not found in the reaction mixtures.

Treatment of corn roots with 2,4-D resulted in an increased utilization of pentose (Table 1), with the increase ranging from 8 to 32 percent in Experiments 1 through 5. The formation of heptulose also was increased from 7 to 70 percent by 2,4-D treatment. The hexose formed was increased from 8 to 122 percent. Thus, in cell-free extracts from the roots of 2,4-D treated corn seedlings, the utilization of pentose and the formation of heptulose and hexose was enhanced.

TPN was added routinely to the reaction mixture although, theoretically, it should not be required to form heptulose and hexose from pentose. In work preliminary to the results reported herein, the effect of TPN on the reaction was studied. No effect was noted, with or without TPN, on the formation of heptulose and hexose; but since the general procedure of Clayton (31) was being used, TPN was added routinely. From the work with G-3-P presented later, no GPDH activity could be demonstrated in cell-free extracts, which supported the idea that the TPN in the reaction mixture was not necessary.

As an indication of the completeness of the procedures used in assaying for sugars, the number of umoles of carbon atoms utilized as pentose and used to form heptulose and hexose was calculated from Table 1. Table 2 contains this accounting of the umoles of carbon atoms utilized as pentose and found in heptulose and hexose, following the 60-minute incubation period. In the buffer treated extracts, from 53 to 99 percent of the carbon atoms are accounted for in hexose and heptulose, with an average of 79 percent. This 79 percent of the carbon atoms compares well with the average of 84 percent in untreated tissue (Experiment 7). In the 2,4-D treated extracts, from 74 to 97 percent of the carbon atoms were accounted for as hexose and heptulose, with an average of 84 percent. This 84 percent accounting

TABLE 2

DISTRIBUTION OF CARBON ATOMS FROM RIBOSE-5-PHOSPHATE FOLLOWING 60-MINUTE INCUBATION PERIOD*

Experiment Number	Treatment**	Micromoles of Carbon Atoms in 6 ml				% of Total Carbon Atoms Accounted for
		Utilized as Pentose	Used to form Heptulose	Used to form Hexose	Unaccounted	
1	Buffer	43.5	28.7	11.4	3.4	92.0
	2,4-D	57.5	37.1	18.6	1.8	97.0
2	Buffer	45.5	30.1	15.0	0.4	99.0
	2,4-D	57.0	32.2	16.8	8.0	86.0
3	Buffer	46.0	27.3	10.2	8.5	82.0
	2,4-D	67.0	30.1	23.4	13.5	80.0
4	Buffer	48.5	13.3	12.6	22.6	53.0
	2,4-D	62.0	26.6	25.8	9.6	58.0
5	Buffer	53.0	20.3	17.4	15.3	71.0
	2,4-D	67.0	30.8	18.6	17.6	74.0
6	Buffer***	12.0	0.0	4.2	7.8	35.0
	2,4-D****	20.5	3.5	5.4	11.6	43.0
7	Untreated	49.5	22.2	13.2	13.9	72.0
	Untreated****	47.0	30.8	13.8	2.4	95.0

*For reaction mixture see Table 1.

**See Table 1.

***Acetone powder extracts.

****2,4-D was added to reaction mixture to a final concentration of 3.3×10^{-5} M.

of carbon atoms is the same as the average of 84 percent with untreated tissue (Experiment 7).

Acetone powders were not as active as cell-free extracts, and the carbon atoms were not as well accounted for as in the reaction mixtures with cell-free extracts (Experiment 6, Tables 1 and 2).

The variability of the results reported in Tables 1 and 2 was a matter of concern. Several factors which may have contributed to this variability are given below. First, there was the inherent difference in separate batches of seedlings. Second, an intermediate compound, R-5-P, of the pentose phosphate pathway, a complex system, was used as the substrate. R-5-P and its products can undergo many reactions to form a large number of substances which were not measured in these studies. At zero time the pentose measured was R-5-P, but at 60 minutes, the pentose could be any or all of the following: R-5-P, ribulose-5-phosphate, xyulose-5-phosphate or xylose-5-phosphate. The variability in heptulose and hexose concentrations may be explained on the basis that they are intermediates in a complex system. Third, it should be noted that when the concentrations of sugar were placed on a basis of per mg N, the amount of variation changed. In the buffer treatment in Experiment 4, the poor accounting of carbon atoms could be explained by the probable formation of three and four-carbon sugars and the two-carbon component glycoaldehyde, none of which were measured.

Chromatographic studies on pentose disappearance. These studies were designed to support the studies on the disappearance of R-5-P. Chromatographic studies following the 60-minute incubation period demonstrated the presence of ribose and sedoheptulose in the reaction mixtures. Known ribose samples had an Rf value of 0.57 and developed a pink color when sprayed with B-naphtholamine. One unknown sugar was identified as ribose by virtue of a similar Rf value and pink color. The known sedoheptulose sample had an Rf of 0.55 and developed a blue color when sprayed with orcinol. An unknown sugar was identified as sedoheptulose by virtue of its Rf value of 0.52 and the development of a blue color when sprayed with orcinol.

Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase was found in extracts from both treated and untreated tissues. The activity of glucose-6-phosphate dehydrogenase is given in Table 5 on the basis of the change in O.D. per mg protein per five minutes, as measured with a Beckman DU spectrophotometer at 340 mu.

Spectrophotometric assay for this enzyme was complicated by the formation of 6-PGA which was the substrate for 6-phosphogluconate dehydrogenase which also reduced TPN, and thereby increased the observed O.D. values. The observed O.D. values were used to calculate the maximum amount of 6-PGA which could be formed in the reaction time. In a separate experiment, this amount of 6-PGA was used as the substrate in the same reaction mixture. Only a

TABLE 3

ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE*

Experiment Number	Treatment**	Change in O.D. per mg Protein per 5 min.	% Increase Due to 2,4-D
8	Buffer	0.70	----
	2,4-D	0.90	28.6
9	Buffer	0.64	----
	2,4-D	0.86	34.4
10	Buffer	0.92	----
	2,4-D	1.06	15.2

*Incubation mixture consisted of: TPN, 400 ug; $MgCl_2$, 10 uM; G-6-P, 10 uM; Tris buffer, pH 7.4, 200 uM; 0.2 ml of cell-free extract; and water to 3.0 ml total volume. O.D. readings at 340 mu.

**See Table 1.

slight change in O.D. could be detected; therefore, the presence of 6-PGA and 6-PGA dehydrogenase was not considered in calculating the activity of glucose-6-phosphate dehydrogenase.

The activity of glucose-6-phosphate dehydrogenase was enhanced by 2,4-D treatment from 15.2 to 34.4 percent over the activity in buffer treated extracts (Table 3). Figure 2 includes a graph of typical data showing the activity of glucose-6-phosphate dehydrogenase in cell-free extracts from both 2,4-D and buffer treated tissue. Buffer treatment resulted in an average change of 0.75 in O.D. per mg protein per five minutes, while the 2,4-D treatment resulted in an average change of 0.94, thus indicating that *in vitro* G-6-P is oxidized faster following 2,4-D treatment.

6-Phosphogluconate dehydrogenase. This enzyme was active in cell-free extracts from both buffer and 2,4-D treated tissue. Table 4 contains the results of assays for this enzyme on a basis of the change in O.D. per mg of protein per five minutes, and the results of a representative experiment are presented in Figure 3.

In assaying for this enzyme, aliquots were used from the same cell-free extracts employed in assaying glucose-6-phosphate dehydrogenase. In all experiments, the activity of 6-phosphogluconate dehydrogenase was higher than that of glucose-6-phosphate dehydrogenase. The buffer treated extracts averaged an O.D. change of 0.98 as

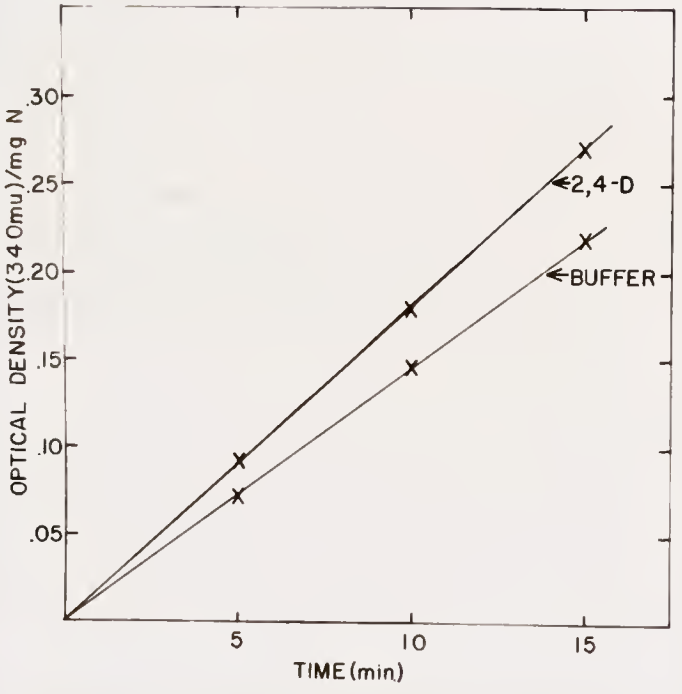


Figure 2. THE REDUCTION OF TPN BY GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN CELL-FREE EXTRACTS. The reaction mixture is given in Table 3.

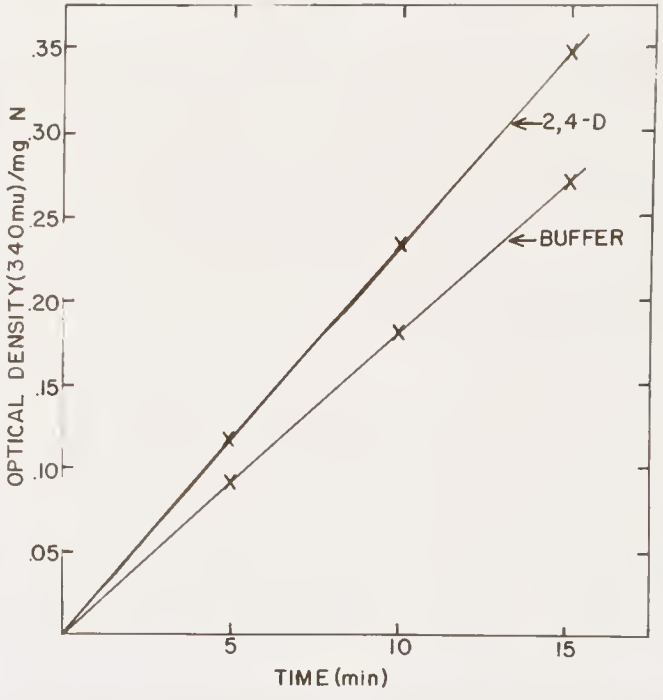


Figure 3. THE REDUCTION OF TPN BY 6-PHOSPHOGLUCONATE DEHYDROGENASE IN CELL-FREE EXTRACTS. The reaction mixture is given in Table 4.

TABLE 4

ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE*

Experiment Number	Treatment**	Change in O.D. per mg Protein per 5 min.	% Increase Due to 2,4-D
11	Buffer	0.89	----
	2,4-D	1.15	29.2
12	Buffer	0.88	----
	2,4-D	1.11	26.1
13	Buffer	1.17	----
	2,4-D	1.26	7.7

*Incubation mixture consisted of: TPN, 400 ug; $MgCl_2$, 10 uM; 6-PGA, 10 uM; Tris buffer, pH 7.4, 200 uM; 0.2 ml of cell-free extract; and water to 3.0 ml total volume. O.D. readings at 340 mu.

**See Table 1.

compared to 0.75, and the 2,4-D treated extracts averaged 1.17 as compared to 0.94. The increase in O.D. of 6-phosphogluconate dehydrogenase over glucose-6-phosphate dehydrogenase was 0.23 in both 2,4-D and buffer treated extracts. Thus it is evident that *in vitro*, 6-PGA is oxidized faster in 2,4-D treated extracts than in buffer treated extracts.

These *in vitro* studies have demonstrated that treatment of corn seedlings with 2,4-D prior to preparing cell-free extracts results in a general enhancement of the activity of the pentose phosphate pathway. This enhancement is evidenced in an increased utilization of R-5-P, an increased formation of heptulose and hexoses from R-5-P, and an increased rate of oxidation of both G-6-P and 6-PGA in cell-free extracts from 2,4-D treated corn seedlings.

Studies of the Glycolytic Pathway

Phosphoglucoisomerase. Phosphoglucoisomerase was active in cell-free extracts from both buffer and 2,4-D treated corn roots (Table 5). The number of umoles of F-6-P produced from G-6-P by phosphoglucoisomerase per mg of nitrogen is given in Table 5. Although 2,4-D lowered the umoles of F-6-P produced in all three experiments, this decrease was not more than seven percent after 20 minutes in the highest case (Experiment 14). These decreases are easily within the range of experimental error.

TABLE 5

ACTIVITY OF PHOSPHOGLUCOISOMERASE*

Experiment Number	Treatment**	uM of F-6-P produced per mg N		
		10 min.	15 min.	20 min.
14	Buffer	63.4	97.6	117.1
	2,4-D	60.9	82.6	108.7
15	Buffer	55.5	116.7	138.8
	2,4-D	66.7	88.9	133.3
16	Buffer	52.8	72.0	107.2
	2,4-D	50.0	60.0	101.9

*The reaction mixtures were composed of: Tris buffer, pH 9.0, 10 uM; G-6-P, 2.5 uM; 0.05 ml of cell-free extract; and to 0.5 ml total volume with water. These were incubated at 38°C for the prescribed periods of time.

**See Table 1.

Therefore, from the results of these in vitro studies with cell-free extracts, 2,4-D treatment does not affect the activity of phosphoglucoisomerase.

6-Phosphofructokinase. Quantitative measurements of the activity of 6-phosphofructokinase were obtained by incubation of F-6-P, ATP and $MgCl_2$ in the presence of aldolase, DPNH and alphaglycerophosphate dehydrogenase. 6-phosphofructokinase was active in cell-free extracts from both untreated and treated tissues (Table 6). The endogenous activity of the reaction mixture was determined and used to calculate the activity of 6-phosphofructokinase. By subtracting the endogenous activity of 0.06 per minute from the activity of the reaction mixture including the cell-free extracts, the activity of 6-phosphofructokinase in the extracts was determined. Table 6 presents the results of these experiments, corrected for endogenous, on the basis of the change in O.D. per mg N per minute.

Treatment with 2,4-D lowered the activity of 6-phosphofructokinase as compared to the activity of the enzyme in cell-free extracts from buffer treated roots. The inhibition of activity ranged from 7.0 to 12.7 percent. Although this inhibition appeared to be small, it was consistently observed.

Aldolase. Aldolase activity in cell-free extracts was measured by trapping the triose phosphates formed when F-1,6-P is cleaved by aldolase. In extracts from both

TABLE 6

ACTIVITY OF 6-PHOSPHOFRUCTOKINASE*

Experiment Number	Treatment**	Change in O.D. per mg N per min.***	% Decrease Due to 2,4-D
17	Buffer	0.63	----
	2,4-D	0.55	12.7
18	Buffer	0.86	----
	2,4-D	0.80	7.0
19	Buffer	0.55	----
	2,4-D	0.48	12.7

*Complete reaction mixture contained: Tris buffer, pH 8.0, 100 uM; ATP, 30 uM; F-6-P, 20 uM; $MgCl_2$, 3 uM; cysteine-hydrochlorid, 20 uM; DPNH, 220 ug; aldolase, 10 ug; alpha-glycerophosphate dehydrogenase, 50 ug; and water to 3.0 ml in corex Beckman cells with 1 cm light path. O.D. readings at 340 mu.

**See Table 1.

***Corrected for endogenous.

control and treated roots, aldolase activity was indicated by the formation of triose phosphate hydrazones (Table 7). Figure 4 pictures the results of a typical experiment, and Table 7 summarizes the results of each experiment. Aldolase activity in cell-free extracts from 2,4-D treated roots was consistently decreased about 12 percent as compared to extracts from buffer treated roots.

Glyceraldehyde-3-phosphate dehydrogenase. Attempts to demonstrate GPDH activity in cell-free extracts were not successful; however, acetone powder extracts from the roots of both buffer and 2,4-D treated seedlings contained a DPN-dependent enzyme. The lack of a TPN-dependent dehydrogenase is in agreement with early work (65) which failed to demonstrate this enzyme in etiolated plant tissue, or tissue which lacked chlorophyll (59), and with the observation of Marcus (103), that TPN triose phosphate dehydrogenase is controlled by the photomorphogenic reaction.

Neither DPNH nor TPNH was oxidized in incubation mixtures containing all of the constituents used to study the reduction of DPN.

Neither cysteine nor glutathione was necessary as a co-factor in the reaction. Other workers have reported that one or the other was necessary (59,96,146,153). The only effect noted when these were included in the reaction mixtures was that the O.D. readings more constantly produced a straight line. Glutathione was added routinely

TABLE 7

ACTIVITY OF ALDOLASE*

Experiment Number	Treatment**	Klett Readings***	% Decrease Due to 2,4-D
20	Buffer	69	--
	2,4-D	60	13
21	Buffer	66	--
	2,4-D	59	11
22	Buffer	67	--
	2,4-D	59	12

*The following reaction mixture was incubated at 38°C for 10 min., stopped with 0.75 N NaOH, and the triose phoshpate hydrazones measured: Tris buffer, pH 8.6, 100 uM; F-1, 6-P, 12.5 uM; hydrazine, 55 uM; enzyme extract; and water to total volume of 2.5 ml.

**See Table 1.

***Change in Klett readings per 0.1 mg of protein added to the reaction mixture.

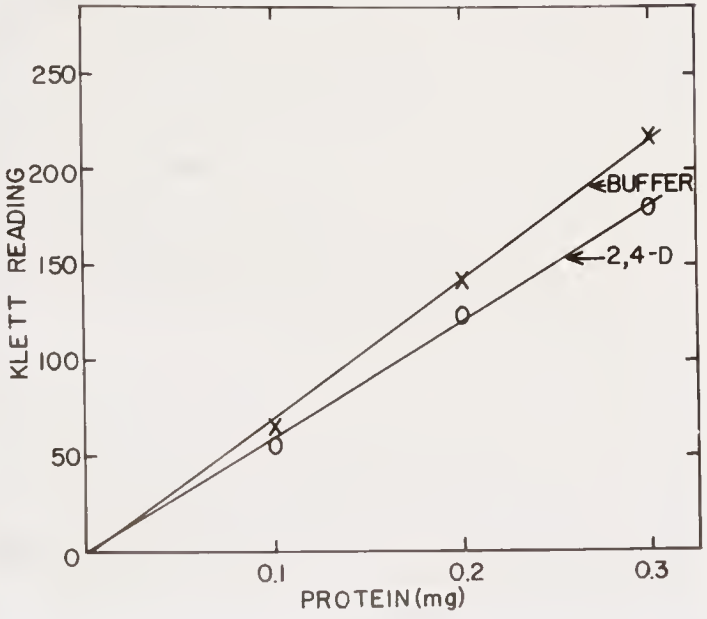


Figure 4. ALDOLASE. The change in Klett reading per mg of protein in cell-free extracts added to the reaction mixture which is given in Table 7.

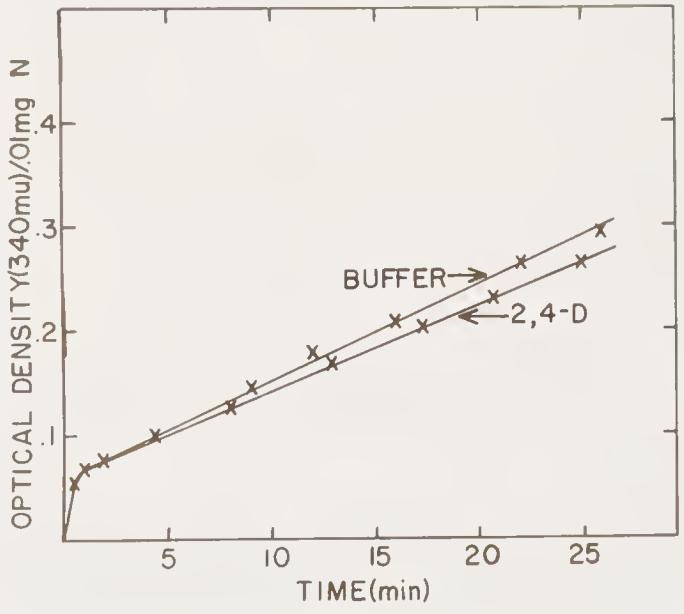


Figure 5. THE REDUCTION OF DPN BY GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN ACETONE POWDER EXTRACTS. The reaction mixture is given in Table 8.

although it was not required. Table 8 shows the results of these experiments and Figure 5 presents a graphical representation of a typical experiment.

The activity of GPDH was decreased from 12 to 20 percent by 2,4-D treatment as compared to extracts from buffer treated corn root. The rates of these reactions were calculated from the linear portion of the graphs, as depicted in Figure 5, in the time intervals after the first two minutes.

Within the first two minutes after starting these reactions by the addition of either enzyme extract or substrate (G-3-P), an extremely rapid increase in O.D. was observed in all cases. This initial increase was immediately followed by a decreased rate which followed a linear pattern as depicted in Figures 5 and 6. In Figure 6, it is shown that this rapid increase can be obtained upon the addition of another aliquot of enzyme extract. This step-like pattern can be repeated numerous times by the addition of aliquots of enzyme extract. The same pattern was obtained with either buffer or 2,4-D treated enzyme extracts. The rapid increase in O.D. immediately following the addition of an aliquot of enzyme extract is the same following each addition of extract (0.065 per 30 seconds). The straight line portion of the curve following the initial increase after the first addition of an aliquot of enzyme extract is a change in O.D. of 0.0063 per 30 seconds, while following the second addition

TABLE 8

ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

Experiment Number	Treatment**	Change in O.D. per mg N per 5 min.	% Decrease Due to 2,4-D
23	Buffer	0.48	--
	2,4-D	0.42	13
24	Buffer	0.56	--
	2,4-D	0.45	20
25	Buffer	0.61	--
	2,4-D	0.54	12

*The reaction mixture contained: Tris buffer, pH 8.5, 150 uM; DPN, 800 ug; sodium arsenate, 170 uM; potassium flouride, 10 uM; reduced glutathione, 15 uM; glyceraldehyde-3-phosphate, 2000 ug; water to 3.0 ml total volume; and acetone powder extract. Blanks omitted DPN. Enzyme extracts were added to start the reaction and the increase in O.D. at 340 mu was followed at timed intervals.

**See Table 1.

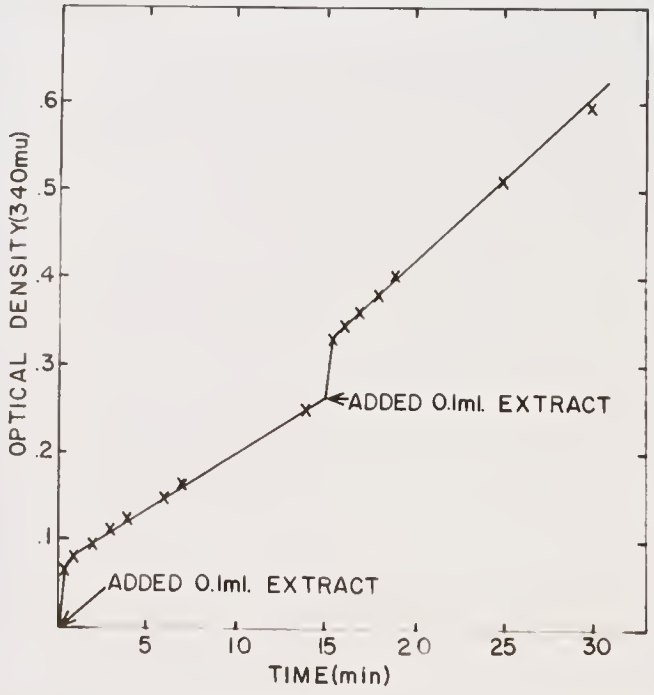


Figure 6. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. The effect of the addition of aliquots of acetone powder extract from buffer treated roots on the reduction of DPN. The reaction mixture is given in Table 8.

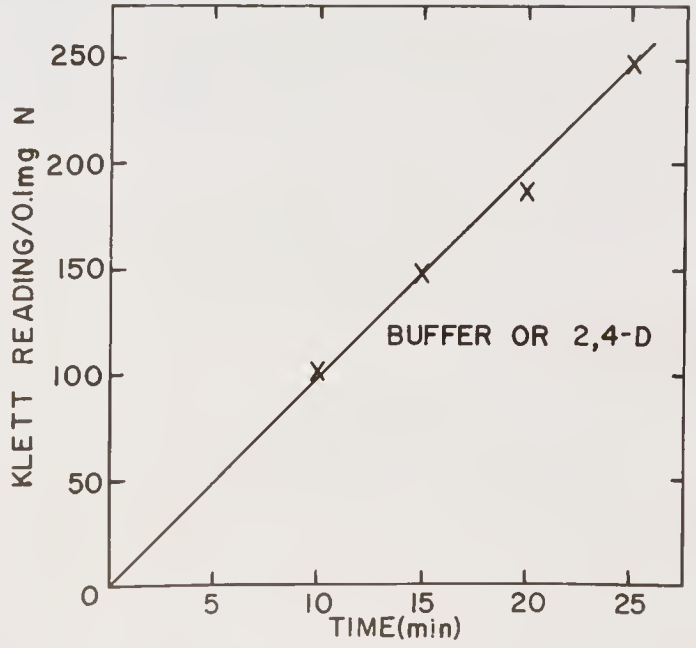


Figure 7. PHOSPHOGLYCERIC KINASE. The change in Klett reading per mg of N in cell-free extracts added to the reaction mixture which is given in Experimental Procedure.

of an aliquot of enzyme extract, a change in O.D. of 0.0094 per 30 seconds was obtained (Figure 6).

The rapid increase in O.D. immediately following the addition of an aliquot of extract or the addition of substrate may be explained on the basis of a rapid reduction of GPDH bound DPN. GPDH has been shown by several workers, with the enzyme from mammalian and yeast tissues (146,153, 154), to combine with DPN in a ratio of about 2 moles of DPN per mole of enzyme. Apparently both DPNH and DPN are bound by the enzyme and both have different dissociation constants. Stockwell (146) states that DPNH has about one tenth of the affinity of DPN for binding sites on the enzyme. Other work suggests that DPN is strongly bound. Thus the slower linear rate after about two minutes of reaction time is probably controlled by the rate of dissociation of DPNH. These slower linear rates are not additive with increased aliquots of extracts. This may be explained on the basis of a build-up of DPNH, which competes with DPN for sites on the enzyme. Other components of the reaction mixture, i.e. substrate, phosphate, arseno-3-phosphoglyceric acid or 3-PGA also may be bound, and thus affect the rate of the reaction.

Phosphoglyceric kinase. The activity of phosphoglyceric kinase was studied by trapping 1,3-DPGA with hydroxylamine. Phosphoglyceric kinase was demonstrated in cell-free extracts from both buffer and 2,4-D treated corn seedlings. Figure 7 gives the type of results obtained

in these experiments. Obviously, 2,4-D treatment of corn seedlings did not affect the *in vitro* activity of phosphoglyceric kinase.

Carboxylases. No carboxylase activity could be demonstrated in either cell-free extracts or acetone powder extracts. Using pyruvate as the substrate, no CO_2 was evolved in the two hour incubation period. This information allowed an assay for the activity of phosphoglyceric mutase, enolase and pyruvic kinase by measuring pyruvate.

Phosphoglyceric mutase. This enzyme was demonstrated qualitatively in both acetone powder extracts and cell-free extracts, but none of these experiments were successful in quantitatively demonstrating the presence of phosphoglyceric mutase. No differences were observed in the activities of phosphoglyceric mutase in extracts from either buffer or 2,4-D treated corn seedlings. The results of these experiments were very erratic; therefore, no conclusions will be drawn from them.

Several factors probably contributed to the failure to demonstrate phosphoglyceric mutase quantitatively. One obvious limitation was the use of simple extracts without attempting to purify the enzyme. Second, the product of the enzyme was not measured, but rather pyruvate was measured, which was formed by two additional reactions from 2-PGA. Third, the two additional reactions also were catalyzed by endogenous enzymes, thus one or both of these enzymes could control the entire chain of reactions.

Enolase. In the experiments designed to study the activity of pyruvic kinase, phosphatase activity in the enzyme preparations was high. Phosphatase catalyzes the conversion of PEP to pyruvate. Therefore, pyruvate was the compound which was measured to determine the activity of enolase. Enolase was active in extracts from both buffer and 2,4-D treated tissue. A pyruvate standard was not made; therefore, for comparison, Klett readings per mg protein added are presented in Table 9.

The rate of enolase activity was not affected by 2,4-D treatment as indicated in Table 9. Identical Klett readings were obtained from both 2,4-D and buffer treated extracts following the ten-minute incubation period. Similar results, not presented, also were obtained using acetone powder extracts.

Pyruvic kinase. McCollum et al. (105,106) reported that pyruvic kinase activity in cell-free extracts from corn seedlings was labile and lost by dialysis, but they successfully demonstrated the enzyme when they used extracts from acetone powders which were not dialyzed. Similar lability of the enzyme was noted in these experiments.

In these studies with acetone powder extracts, a high endogenous phosphatase activity was noted in the reaction mixtures minus ADP which made measurement of this enzyme difficult. Inclusion of ADP in the reaction mixture did not increase the pyruvate formation above the endogenous

TABLE 9

ACTIVITY OF ENOLASE*

Experiment Number	Treatment**	Klett readings per mg protein
26	Buffer	20
	2,4-D	20
27	Buffer	36
	2,4-D	37
28	Buffer	27
	2,4-D	26

*The reaction mixture contained: Tris buffer, pH 7.4, 150 uM; 2-PGA, 4500 ug; MgCl 20 uM; up to 0.4 ml of cell-free extract; and water to 3.0 ml total volume.

**See Table 1.

except in a few cases. When the addition of ADP increased the formation of pyruvate, the increase did not in any case exceed the endogenous in the buffer treated extracts over 25 percent and the 2,4-D treated extracts over eight percent. Thus, buffer extracts indicated a higher pyruvic kinase activity than 2,4-D extracts. Attempts to quantitatively measure the rate of pyruvate formation in both types of extracts were not successful. This may be explained on the basis of the lack of purity of the enzyme extracts, enzyme lability and the high endogenous phosphatase activity.

These *in vitro* studies of the enzymes of the glycolytic pathway indicate that 2,4-D treatment of etiolated corn seedlings decreases the activity of 6-phosphofructokinase, aldolase and glyceraldehyde-3-phosphate dehydrogenase. The activity of pyruvic kinase, although not quantitatively measured, also was slightly decreased following 2,4-D treatment. At the same time, the activities of phosphoglucoisomerase, phosphoglyceric kinase and enolase were similar in extracts from both buffer and 2,4-D treated tissue. Studies of phosphoglyceric mutase were inconclusive.

DISCUSSION

In a study of the *in vitro* activity of enzymes, several inherent problems must be recognized. These problems are concerned with definite factors which can influence the observed results; therefore, they should at least be stated and kept in mind when the results are discussed and interpreted.

Several assumptions were made in these studies which should be noted. First, the assumption was made that 2,4-D did not affect the extraction of an enzyme or enzymes from the treated tissue. When similar extraction procedures are followed with both treated and untreated tissues, are similar percentages of each enzyme extracted from both tissues? Second, in *in vitro* work, changes could occur in an enzyme or enzymes as a result of the extraction procedure. Note that in the results, the activities of several enzymes from treated and untreated tissues were the same. One interpretation of this might be that the completeness of extraction was the same from both treated and untreated tissues. Third, by the nature of this study it was not possible to purify the enzymes; therefore side reactions and substrate specificity of the enzymes were inherent

problems. A fourth problem is metabolic toxicity or stimulation. The effects of non-physiological substances which are often employed in studying a particular enzyme such as sodium arsenate used in the study of glyceraldehyde-3-phosphate dehydrogenase, is a fifth problem. Sixth, when a specific enzyme was studied in these experiments, the reaction mixture was prepared according to a general procedure which has been worked out for a reasonably pure enzyme. Such factors as temperature, pH and co-factors of the reaction have been determined for the pure enzyme, and it was assumed that these were the required factors for the enzymes in this study, although they were not purified and were from another source. Some other factors which may affect the results are presented later.

As noted in the review of literature, several workers have reported that 2,4-D affects the level of protein-nitrogen in treated tissues. If 2,4-D has this effect, obviously, in a study of this type in which the results are given on the basis of per mg N or per mg protein, this could skew the results. In 50 consecutive N determinations, 2,4-D treated tissues had an increased N content in 20 determinations. In 16 determinations, buffer treated tissues had the highest N, and in 14 determinations, they were equal. The N content varied as much as 20 percent in a few instances, but in no instance did the N determinations affect the trend of the observed results of an experiment.

In this study, if the seedlings were placed in deionized water and soaked for periods above four hours, beginning after the 12-hour treatment of the corn seedlings, the effects of 2,4-D on glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase could be partially reversed.

Apparently when the seedlings were soaked in deionized water, substances or substance were exuded into the soaking solution. Root extracts made following soaking from both buffer and 2,4-D treated seedlings demonstrated similar enzymatic activity. The deionized water used to soak the seedlings was concentrated to about one ml. When aliquots of this concentrated soaking solution were added to the reaction mixtures containing the enzyme extracts, a slight inhibition of enzyme activity was noted. In one experiment, the increase in 2,4-D extracts made prior to soaking was 20.3 percent. Following soaking, the increase was only 4.2 percent. When the concentrated soaking solution was added to the reaction mixtures, the increase was 8.0 percent. Several research workers have reported the exudation of substances from roots which affected growth. Eliasson (39) reported that wheat roots exuded a substance which was inhibitory to the growth of wheat roots. He further noted that washing roots of pea seedlings treated with 2,4-D counteracted the inhibition of root elongation and swelling caused by 2,4-D (38). He concluded that his results indicated that a leakable, growth-inhibitory substance is formed in the roots in the presence of 2,4-D. Fries and

Forsman (53) identified some amino acids and nucleic acid derivatives in pea root exudates. Howell (76) also reported an inhibitory substance in pea roots. Housley et al. (75) extracted several growth-inhibiting and growth-promoting substances from the roots of maize. Soybean seedlings, particularly seedlings treated with 2,4-D, contain a compound which acts as an inhibitor of oxidative phosphorylation (90).

Consideration of this reversal of the effects of 2,4-D by soaking the treated seedlings raises a question concerning another portion of this study. Since soaking intact seedlings will reverse 2,4-D effects, why doesn't dialysis of the extracts have similar effects? In this connection it should be noted that glyceraldehyde-3-phosphate dehydrogenase was studied in acetone powder extracts which were not dialyzed. Thus, the reversal of the effects of 2,4-D by soaking intact tissue was noted on both dialyzed cell-free extracts and undialyzed acetone powder extracts.

The results of this study support the observations by Humphreys and Dugger (80,81,82) concerning the catabolism of glucose in intact corn roots. Their *in vivo* work suggested that 2,4-D treatment of etiolated corn seedlings affected glucose catabolism through an increase in the amount of glucose catabolized via the pentose phosphate pathway. This *in vitro* study of the enzyme activity of both the pentose phosphate pathway and the glycolytic

pathway indicates that 2,4-D treatment enhances the activity of the pentose phosphate pathway enzymes. At the same time, the activities of some glycolytic enzymes were inhibited following 2,4-D treatment.

The results of these studies clearly raise the question of whether or not the observed changes in enzyme activity are of sufficient magnitude to affect the pathways of glucose metabolism in intact tissues. Since both of the major catabolic pathways of glucose were demonstrated in both buffer and 2,4-D treated tissues, why should the pentose phosphate pathway be favored over the glycolytic pathway? It is difficult to see why such small changes can affect the metabolism of a plant. Note should be taken of the fact that glycolytic enzymes were more difficult to demonstrate and that acetone powder had to be used in some cases, i.e. glyceraldehyde-3-phosphate dehydrogenase.

Two groups of research workers recently reported on regulatory mechanism of carbohydrate metabolism. Racker and co-workers (57,58,169,170,171) have worked extensively on the regulatory mechanisms in carbohydrate metabolism via the glycolytic pathway in reconstructed systems, in ascites tumor cells and HeLa cells. Chance and Hess (24,25,26,27, 28,29,72) also have studied metabolic control mechanisms using ascite tumor cells and bakers' yeast cells.

Wu and Racker (170) found that enzymes are present in ascites tumor cell extracts in sufficient quantities to account for glucose utilization via glycolysis in intact

cells. Under certain conditions, they noted that glycolysis was inhibited even though sufficient quantities of enzymes were present. They postulated that some co-factor or co-factors may be limiting the glycolytic pathway. They demonstrated that the intracellular concentration of inorganic phosphate fluctuated parallel to the rate of glycolysis. Thus, they concluded that the concentration of inorganic phosphate was a major limiting factor in glycolysis.

In this series of experiments, the enzymes of both glycolysis and the pentose phosphate pathway were present in the extracts. Even though previous in vivo work indicated that 2,4-D treatment caused a shift of glucose catabolism to the pentose phosphate pathway, and this work supports this as indicated by the stimulation of the pentose phosphate pathway and the inhibition of glycolysis, it is difficult to explain the action of 2,4-D on the basis of the enzyme activity of the two pathways. Since the enzymes of both pathways are present, it is proposed that a co-factor or co-factors may be limiting glycolysis, or that the co-factors may be stimulating the pentose phosphate pathway. Thus, the actual presence of an enzyme may not mean that the enzyme is active, but rather, that if the proper co-factors are present, the enzyme is active.

Two major types of co-factors are affected by 2,4-D. First 2,4-D affects the ion concentration; and second, it indirectly affects nucleotides in various fashions.

Inorganic phosphate is a well-known co-factor of glycolysis. Racker and co-workers (57,58,169,170,171) demonstrated that inorganic phosphate may be a limiting co-factor, and thus exert some metabolic control. Theorell (150) demonstrated in 1935, that inorganic phosphate inhibited glucose-6-phosphate dehydrogenase. Kravitz and Guarino (95), in 1958, using an enzyme extract from ascites tumor cells, also demonstrated that inorganic phosphate inhibited the activity of glucose-6-phosphate dehydrogenase. These observations could be interpreted to indicate that with high inorganic phosphate content, glycolysis is stimulated and the pentose phosphate pathway is inhibited, while with low inorganic phosphate concentrations, the pentose phosphate pathway is stimulated. Thus, experimental results, such as those of Ormrod (118), in which the inorganic phosphate dropped sharply within five minutes, could indicate a shift to the pentose phosphate pathway. 2,4-D possibly affects other ions, but only inorganic phosphate has been shown experimentally to affect the metabolic control mechanisms.

Kravitz and Guarino (95) found that the pentose phosphate pathway could be stimulated by additions of TPN or of electron acceptors for reduced TPN. So any action whereby TPN is formed, either by synthesis or by oxidation of reduced TPN, may stimulate the pentose phosphate pathway. Action of growth regulators through nucleic acid metabolism was proposed and somewhat

substantiated by Skoog (136,139,140). Key et al. (92), working with 2,4-D treated soybeans, concluded that 2,4-D may affect the metabolism of nucleotides which are involved in mitochondria growth. In certain 2,4-D treated tissue, the mitochondria were swollen and their acid-soluble nucleotide content increased. They concluded that growth induced by auxins involves a growth of mitochondria, and they postulated that this growth is regulated through nucleotide metabolism. Stimulation of the pentose phosphate pathway by 2,4-D can be correlated with the synthesis of nucleotides. In studies on ribose metabolism, Hiatt and Lareau (73) recently demonstrated that labeled glucose fed to tissues which were oxidizing glucose via the pentose phosphate pathway resulted in the synthesis of labeled ribose and of nucleotides in which the sugar moiety was labeled similarly. Thus, the increased utilization of pentose in these experiments could result in an increased nucleotide synthesis which could affect the metabolic control mechanism. Sie et al. (135) recently described a system whereby sedoheptulose monophosphate is formed from purine and pyrimidine nucleotides. If these reactions are assumed to occur in plant tissues and to be reversible, then the increased formation of sedoheptulose in 2,4-D treated extracts, which was observed in these experiments, could be related to nucleotide metabolism, and thus to a metabolic control mechanism.

Thus, experimental data support the theory that 2,4-D could affect the metabolic control mechanisms. The elucidation of this mechanism will be dependent upon further research employing such research techniques as those of Chance and Hess (30), whereby small differences can be measured accurately in short-time intervals employing intact tissues.

SUMMARY AND CONCLUSIONS

A series of experiments was conducted to study the effects of 2,4-D on the *in vitro* activity of enzymes extracted from etiolated corn roots in an attempt to determine the mechanism whereby 2,4-D shifted the normal pathway of glucose catabolism. Comparisons were made between the activities of enzymes extracted from the roots of both buffer and 2,4-D treated etiolated corn seedlings.

Three-day-old etiolated corn seedlings were divided into two groups and treated either with 10^{-2} M phosphate buffer (pH 5.3), or with buffer plus 10^{-3} M 2,4-D for 12 hours at 22°C. After the 12-hour treatment period, the seedlings were removed, washed, blotted dry and the roots excised. The roots were weighed and used to prepare enzyme extracts. Cell-free extracts and acetone powder extracts were prepared from each group of seedlings. The activities of the enzymes of both the glycolytic and the pentose phosphate pathway were studied, employing either cell-free extracts or acetone powder extracts as the enzyme source. Individual enzymes were studied in reaction mixtures containing excess substrate plus the known cofactors, with enzyme concentration being

the limiting factor of the reaction. The activity of each enzyme was determined in extracts from both buffer and 2,4-D treated tissues.

The results of these studies justify the following conclusions:

1. Glycolytic enzymes and the enzymes of the pentose phosphate pathway were present in extracts from roots of both untreated and 2,4-D treated corn seedlings.

2. The activities of 6-phosphofructokinase, aldolase and glyceraldehyde-3-phosphate dehydrogenase were decreased in extracts from 2,4-D treated tissue, while the activities of phosphoglucoisomerase, phosphoglyceric kinase and enolase were not affected. Studies of phosphoglyceric mutase were not conclusive.

3. The activities of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase were enhanced in extracts from 2,4-D treated tissue.

4. In extracts from 2,4-D treated tissue, there was an increased utilization of R-5-P and an increased formation of heptulose and hexose.

5. These *in vitro* studies support the *in vivo* observation that 2,4-D treatment of etiolated corn seedlings affects glucose catabolism through an increase in the amount of glucose catabolized via the pentose phosphate pathway.

6. A theory is presented and discussed which proposes that 2,4-D in some manner effects the normal metabolic control mechanisms of intact cells.

BIBLIOGRAPHY

1. Adamson, D. and H. Adamson. Auxin action on coleoptiles in the presence of nitrogen and at low temperature. Science 128:532-533. 1958.

2. Akers, T. J. and S. C. Fang. Studies in plant metabolism. VI. Effect of 2,4-D on the metabolism of aspartic acid and glutamic acid in the bean plant. Plant Physiol. 31:34-37. 1956.

3. Armarego, W. L. F., A. J. Canny and S. F. Cox. Metal-chelating properties of plant-growth substances. Nature 183:1176-1177. 1959.

4. Asana, R. D., G. Verma and V. S. Rani. Some observations on the influence of 2,4-dichlorophenoxyacetic acid (2,4-D) on the growth and development of two varieties of wheat. Physiol. Plant. 3:334-352. 1950.

5. Ashwell, G. Cysteine-H_2SO_4 reaction for hexose. In Methods in Enzymology III. S. P. Colowick and N. O. Kaplan, Ed. pp. 81-82. New York: Academic Press. 1957.

6. Avery, G. S., Jr. Stimulation of respiration in relation to growth. In Plant Growth Substances. F. Skoog, Ed. pp. 105-109. Madison, Wisconsin: University of Wisconsin Press. 1951.

7. Axelrod, B. and R. S. Bandurski. Phosphoglyceryl kinase in higher plants. J. Biol. Chem. 204: 939-948. 1953.

8. Bail, A. Die Diagnose der Pentosurie. Deut. med. Woch. 28:253-254. 1902.

9. Bass, S. T., C. L. Hammer and H. M. Sell. Effects of 2,4-dichlorophenoxyacetic acid on the mineral contents of cranberry bean plants (Phaseolus vulgaris). Mich. State Univ. Agr. Exp. Sta. Quart. Bull. 42:43-46. 1959.

10. Beck, W. S. Determination of triose phosphate and proposed modifications in the aldolase method of Sibley and Lehninger. J. Biol. Chem. 212:847-857. 1955.

11. Bennet-Clark, T. A. Salt accumulation and mode of action of auxin. In Chemistry and Mode of Action of Plant Growth Substances. R. L. Wain and F. Wightman, Ed. pp. 284-291. New York: Academic Press. 1956.

12. Berg, R. T. and L. W. McElroy. Effect of 2,4-D on the nitrate content of forage crops and weeds. Can. J. Agric. Sci. 33:354-358. 1953.

13. Berger, J. and G. S. Avery, Jr. Glutamic and isocitric acid dehydrogenases in the Avena coleoptile and the effect of auxins on these enzymes. Amer. J. Bot. 31:11-19. 1944.

14. Berger, J. and G. S. Avery, Jr. The mechanism of auxin action. Science 98:454-455. 1943.

15. Biswas, B. B. and S. P. Sen. Relationship between auxins and nucleic acid synthesis in coleoptile tissues. Nature 183:1824-1825. 1959.

16. Bonner, J., R. S. Bandurski and A. Millerd. Linkage of respiration to auxin induced water absorption. Physiol. Plant. 6:511. 1953.

17. Boroughs, H. and J. Bonner. Effects of indoleacetic acid on metabolic pathways. Arch. Biochem. Biophys. 46:279-290. 1953.

18. Brakke, M. K. and L. G. Nichell. Lack of effect of plant growth-regulators on the action of alpha-amylase secreted by virus tumor tissue. Bot. Gaz. 113:482-484. 1952.

19. Brody, T. M. Effect of certain plant growth substances on oxidative phosphorylation in rat liver mitochondria. Proc. Soc. Exptl. Biol. Med. 80: 533-536. 1952.

20. Brown, J. W. Effect of 2,4-D on the H_2O relations, the accumulation and distribution of solid matter, and the respiration of bean plants. Bot. Gaz. 107:332-343. 1946.

21. Bryan, W. H. and E. H. Newcomb. Stimulation of pectin methylesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plant. 7:290. 1954.

22. Burstrom, H. Studies on growth and metabolism of roots. V. Cell elongation and dry matter content. Physiol. Plant. 4:199-207. 1951.

23. Carr, D. J. and E. K. Ng. Residual effects of auxin, chelating agents, and metabolic inhibitors in cell extension. Aust. J. Biol. Sci. 12:373-387. 1959.

24. Chance, B. Phosphorylation efficiency of the intact cell. II. Crossover phenomena in Baker's yeast. J. Biol. Chem. 234:3036-3040. 1959.

25. Chance, B. Phosphorylation efficiency of the intact cell. III. Phosphorylation and dephosphorylation in yeast cells. J. Biol. Chem. 234:3041-3043. 1959.

26. Chance, B. and B. Hess. Metabolic control mechanisms. I. Electron transfer in the mammalian cell. J. Biol. Chem. 234:2404-2412. 1959.

27. Chance, B. and B. Hess. Metabolic control mechanisms. II. Crossover phenomena in mitochondria of ascites tumor cells. J. Biol. Chem. 234:2413-2415. 1959.

28. Chance, B. and B. Hess. Metabolic control mechanisms. III. Kinetics of oxygen utilization in ascites tumor cells. J. Biol. Chem. 234:2416-2420. 1959.

29. Chance, B. and B. Hess. Metabolic control mechanisms. IV. The effect of glucose upon the steady state respiratory enzymes in the ascites cell. J. Biol. Chem. 234:2421-2427. 1959.

30. Chance, B. and B. Hess. Spectroscopic evidence of metabolic control. Science 129:700-708. 1959.

31. Clayton, R. A. Pentose cycle activity in cell-free extracts of tobacco leaves and seedlings. Arch. Biochem. Biophys. 79:111-123. 1959.

32. Cohen, D., B. Ginsburg and C. Heitner-Wirguin. Metal-chelating properties of plant-growth substances. Nature 181:686-687. 1958.

33. Cooke, A. R. Influence of 2,4-D on the uptake of minerals from the soil. Weeds 5:25-28. 1957.

34. Corns, W. G. Effects of 2,4-D and soil moisture on the catalase activity, respiration and protein content of bean plants. Cana. J. Res. 28C:393-405. 1950.

35. Croker, B. H. Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on mitosis in Allium cepa. Bot. Gaz. 114:274-283. 1953.

36. Datta, S. C. and S. Dunn. Effects of light quality on herbicide toxicity to plants. Weeds 7:55-65. 1959.

37. Dische, Z., L. B. Shettles and M. Osnos. New specific color reactions of hexoses and spectrophotometric micromethods for their determination. Arch. Biochem. 22:169-184. 1949.

38. Eliasson, L. Growth response of pea roots to 2,4-D applied to the hypocotyl. Physiol. Plant. 12:834-840. 1959.

39. Eliasson, L. Inhibition of the growth of wheat roots in nutrient solutions by substances exuded from the roots. Ann. Royal Agr. Coll. Sweden. 25:285-293. 1959.

40. Erickson, L. C., C. I. Seeley and K. H. Klages. Effect of 2,4-D upon the protein content of wheats. J. Amer. Soc. Agron. 40:659-660. 1948.

41. Erickson, L. C., R. T. Wedding and B. L. Brannaman. Influence of pH on 2,4-dichlorophenoxyacetic acid and acetic acid activity in Chlorella. Plant Physiol. 30:69-74. 1955.

42. Fang, S. C., F. Tenny and J. S. Butts. Influence of 2,4-dichlorophenoxyacetic acid on pathways of glucose utilization in bean stem tissues. Plant Physiol. 35:405-408. 1960.

43. Fang, S. C. and J. S. Butts. Studies in plant metabolism. IV. Comparative effects of 2,4-dichlorophenoxyacetic acid and other plant growth regulators on phosphorus metabolism in bean plants. Plant Physiol. 29:365-368. 1954.

44. Fawcett, C. H. Plant-growth substances and the copper chelation theory of their mode of action. Nature 184:796-798. 1959.

45. Fischer, E. H. and J. Fellig. Salivary amylase inhibition. Science 115:684-685. 1952.

46. Frank, P. A. and B. H. Grigsby. Effects of herbicidal sprays on nitrate accumulation in certain weed species. Weeds 5:206-217. 1957.

47. Freeland, R. O. Effects of growth substances on photosynthesis. Plant Physiol. 24:621-628. 1949.

48. Freiberg, S. R. Effects of exogenous growth regulator on proteolytic enzymes of the soybean plant. Science 115:674-675. 1952.

49. Freiberg, S. R. and H. E. Clark. Changes in nitrogen fractions and proteolytic enzymes of soybean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 30:39-46. 1955.

50. Freiberg, S. R. and H. E. Clark. Effects of 2,4-dichlorophenoxyacetic acid upon the nitrogen metabolism and water relations of soybean plants grown at different nitrogen levels. Bot. Gaz. 113:322-333. 1952.

51. French, R. C. and H. Beevers. Respiratory and growth responses induced by growth regulators and allied compounds. Amer. J. Bot. 40:660-666. 1953.

52. Friedemann, T. E. and G. F. Haugen. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. 147:415-442. 1943.

53. Fries, N. and B. Forsman. Quantitative determination of certain nucleic acid derivatives in pea root exudates. Physiol. Plant. 4:410-420. 1951.

54. Fults, J. L. and M. G. Payne. Effects of 2,4-dichlorophenoxyacetic acid and maleic hydrazide on free amino acids and proteins in potato, sugar beet, and bean tops. Bot. Gaz. 118:130-133. 1956.

55. Galston, A. W. and R. Kaur. An effect of auxins on the heat coagulability of the proteins of growing plant cells. Proc. Natl. Acad. Sci. 45:1587-1590. 1959.

56. Galston, A. W. and W. K. Purves. The mechanism of action of auxin. Ann. Rev. Plant Physiol. 11:239-276. 1960.

57. Gatt, S. and E. Racker. Regulatory mechanisms in carbohydrate metabolism. I. Crabtree effect in reconstructed systems. J. Biol. Chem. 234:1015-1023. 1959.

58. Gatt, S. and E. Racker. Regulatory mechanisms in carbohydrate metabolism. II. Pasteur effect in reconstructed systems. J. Biol. Chem. 234:1024-1028. 1959.

59. Gibbs, M. Triosephosphate dehydrogenase and glucose-6-phosphate dehydrogenase in the pea plant. Nature 170:164. 1952.

60. Glaszious, K. T. The effect of auxins on the binding of pectin methylesterase to cell wall preparations. Aust. J. Biol. Sci. 10:426-434. 1957.

61. Glasziou, K. T. Effect of 2,4-dichlorophenoxyacetic acid on pectin methylesterase in tobacco pith sections. Nature 181:428-429. 1958.

62. Glaszious, K. T. and S. D. Inglis. The effect of auxin on the binding of pectin methylesterase to cell walls. Aust. J. Biol. Sci. 11:127-141. 1958.

63. Gordon, S. A. Auxin-protein complexes of the wheat grain. Amer. J. Bot. 33:160-169. 1946.

64. Grisolia, S., L. C. Mokrasch and V. D. Hospelhorn. Adenosine mono-, di- and triphosphate, pyruvic kinase, hexokinase and polynucleotide phosphorylase assay. Biochem. Biophys. Acta. 28:350-354. 1958.

65. Hageman, R. H. and D. I. Arnon. Changes in glyceraldehyde phosphate dehydrogenase during the life cycle of a green plant. Arch. Biochem. and Biophys. 57:421-436. 1955.

66. Hagen, C. E., C. O. Clagett and E. A. Helgeson. 2,4-dichlorophenoxyacetic acid inhibition of castor bean lipase. Science 110:116-117. 1949.

67. Hanson, J. B. and J. Bonner. The relationship between salt and water uptake in Jersulem artichoke tuber tissue. Amer. J. Bot. 41:702-710. 1954.

68. Heath, O.V . S. and J. E. Clark. Chelating agents as plant growth substances. A possible clue to the mode of action on auxin. Nature 177:1118-1121. 1956.

69. Heath, O. V. S. and J. E. Clark. Chelating agents as growth substances. Nature 178:600-601. 1956.

70. Heath, O. V. S. and J. E. Clark. Comment on the article by Armarego. Nature 183:1177. 1959.

71. Henderson, J. H. M., I. H. Miller and D. C. Desse. Effect of 2,4-dichlorophenoxyacetic acid on respiration and on destruction of indoleacetic acid in oat and sunflower tissues. Science120:710-712. 1954.

72. Hess, B. and B. Chance. Phosphorylation effeciency of the intact cell. I. Glucose-oxygen titrations in ascites tumor cells. J. Biol. Chem. 234:3031-3055. 1959.

73. Hiatt, H. H. and J. Lareau. Studies of ribose metabolism. VIII. Pathways of ribose biosynthesis in vivo and in vitro in rat, mouse, and human tissues. J. Biol. Chem. 235:1241-1245. 1960.

74. Horecker, B. L. and P. Z. Smyroniotis. The enzymatic formation of sedoheptulose phosphate from pentose phosphate. J. Amer. Chem. Soc. 74:2123. 1952.

75. Housley, S., A. Booth and I. D. J. Phillips. Stimulation of coleoptile and root-growth by extracts of maize. Nature 178:255-256. 1956.

76. Howell, R. W. The inhibiting effect of root tips on the elongation of excised Pisum epicotys cultures in vitro. Plant Physiol. 29:100. 1954.

77. Hsueh, Y. L. and C. H. Lou. Effect of 2,4-D on seed germination and respiration. Science 105:283-285. 1947.

78. Humphreys, T. E. and W. M. Dugger, Jr. The effect of 2,4-dichlorophenoxyacetic acid on the respiration of etiolated pea seedlings. Plant Physiol. 31 Suppl.:xxii. 1956.

79. Humphreys, T. E. and W. M. Dugger, Jr. The effect of 2,4-dichlorophenoxyacetic acid on pathways of glucose catabolism in higher plants. Plant Physiol. 32:136-140. 1957.

80. Humphreys, T. E. and W. M. Dugger, Jr. The effect of 2,4-dichlorophenoxyacetic acid on the respiration of etiolated pea seedlings. Plant Physiol. 32:530-536. 1957.

81. Humphreys, T. E. and W. M. Dugger, Jr. Effect of 2,4-dichlorophenoxyacetic acid and 2,4-dinitrophenol on the uptake and metabolism of exogenous substrates by corn roots. Plant Physiol. 34:112-116. 1959.

82. Humphreys, T. E. and W. M. Dugger, Jr. Use of specifically labeled glucose and gluconate in the evaluation of catabolic pathways for glucose in corn roots. Plant Physiol. 34:580-582. 1959.

83. Johnson, E. J. and A. R. Colmer. Relationship between magnesium and the physiological effects of 2,4-dichlorophenoxyacetic acid on Azotobacter vinelandii and Rhizobium meliloti. Jour. Bact. 73:139-143. 1957.

84. Johnson, E. J. and A. R. Colmer. Further studies on the mode of action of 2,4-dichlorophenoxyacetic acid on Azotobacter vinelandii as related to magnesium and phosphate. Jour. Bact. 73:666-669. 1957.

85. Johnson, E. J. and A. R. Colmer. The relation of magnesium ion to the inhibition of the respiration of Azotobacter vinelandii by aureomycin, achromycin and 2,4-dichlorophenoxyacetic acid. Antibiotics and Chemotherapy 7:521-526. 1957.

86. Johnson, E. J. and A. R. Colmer. The relationship of magnesium ion and molecular structure of 2,4-dichlorophenoxyacetic acid and some related compounds to the inhibition of the respiration of Azotobacter vinelandii. Plant Physiol. 33:99-101. 1958.

87. Kachmar, J. F. and P. D. Boyer. Kinetic analysis of enzyme reactions. I. The potassium activation and calcium inhibition of pyruvic phosphoferase. J. Biol. Chem. 200:669-682. 1953.

88. Kelly, S. and G. S. Avery. The effect of 2,4-dichlorophenoxyacetic acid and other physiologically active substances on respiration. Amer. J. Bot. 36:421-426. 1949.

89. Kelly, S. and G. S. Avery. The age of pea tissue and other factors influencing the respiratory response to 2,4-dichlorophenoxyacetic acid and dinitro compounds. Amer. J. Bot. 38:1-5. 1951.

90. Key, J. L. and D. S. Galitz. Growth inhibitor in immature soybean seeds and 2,4-D sprayed seedlings. Science 130:1339-1340. 1956.

91. Key, J. L. and J. B. Hanson. Accumulation of a naturally occuring growth inhibitor in soybeans following treatment with 2,4-D. Plant Physiol. Suppl.:xvii. 1959.

92. Key, J. L., J. B. Hanson and R. F. Bils. Effect of 2,4-dichlorophenoxyacetic acid application on activity and composition of mitochondria from soybeans. Plant Physiol. 35:177-183. 1960.

93. Klevstrand, R. and A. Nordal. A spraying reagent for paper chromatograms which is apparently specific for ketoheptoses. Acta. Chem. Scand. 4:1320. 1950.

94. Klingman, G. C. and G. H. Ahlgren. Effects of 2,4-D on dry weight, reducing sugars, total sugars, polysaccharides, nitrogen and allyl sulfide in wild garlic. Bot. Gaz. 113:119-134. 1951.

95. Kravitz, E. A. and A. J. Guarino. On the effect of inorganic phosphate on hexose phosphate metabolism. Science 128:1139-1140. 1958.

96. Krimsky, I. and E. Racker. Glutathione, a prosthetic group of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. 198:721-729. 1952.

97. Kvamme, O. J., C. O. Clagett and W. B. Treumann. Kinetics of the action of the sodium salt of 2,4-dichlorophenoxyacetic acid on the germ lipase of wheat. Arch. Biochem. 24:321-328. 1949.

98. Ling, Kuo-Huang, W. L. Byrne and H. Lardy. Phosphohexokinase. In Methods in Enzymology. I. S. P. Colowick and N. P. Kaplan, Ed. pp. 306-308. New York: Academic Press. 1955.

99. Livingstone, C., M. G. Payne and J. L. Fultz. Effects of maleic hydrazide and 2,4-dichlorophenoxyacetic acid on the free amino-acids in sugar beets. Bot. Gaz. 116:148-156. 1954.

100. Loustalot, A. J. and T. J. Muzik. Effect of 2,4-D on apparent photosynthesis and developmental morphology of Velvet Bean. Bot. Gaz. 115:56-66. 1953.

101. Loustalot, A. J., M. P. Morris, J. Garcia and C. Pagan. 2,4-D affects phosphorus metabolism. Science 118:627-628. 1953.

102. Luecke, R. W., C. L. Hammer and H. M. Sell. Effect of 2,4-dichlorophenoxyacetic acid on the content of thiamine, riboflavin, nicotinic acid, pantothenic acid and carotene in stems and leaves of red kidney bean plants. Plant Physiol. 24:546-548. 1949.

103. Marcus, A. Photocontrol of formation of red kidney bean leaf triphosphopyridine nucleotide linked triosephosphate dehydrogenase. Plant Physiol. 35:126-128. 1960.

104. Marre, E. and G. Forti. Metabolic responses to auxin. III. The effects of auxin on ATP level as related to the auxin induced respiration increase. Physiol. Plant. 11:36-47. 1958.

105. McCollum, R. E., R. H. Hageman and E. H. Tyner. Influence of potassium on pyruvic kinase from plant tissue. Soil Science 86:324-331. 1958.

106. McCollum, R. E., R. H. Hageman and E. H. Tyner. Occurance of pyruvic kinase and phosphoenolpyruvate phosphatases in seeds of higher plants. Soil Science 89:49-52. 1960.

107. McElroy, W. C. and B. Glass, Ed. Symposium on phosphorus metabolism. I. Baltimore: Johns Hopkins Press. 1951.

108. McElroy, W. C. and B. Glass, Ed. Symposium on phosphorus metabolism. II. Baltimore: Johns Hopkins Press. 1952.

109. Mejbaum, W. Über die Bestimmung Kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaüre. Z. Physiol. Chem. 258:117-120. 1939.

110. Miller, I. H. and R. H. Burris. Effect of plant growth substances upon oxidation of ascorbic and glycolic acids by cell-free enzymes from barley. Amer. J. Bot. 38:547-549. 1951.

111. Mitchell, J. W. and J. W. Brown. Effect of 2,4-D acid in the readily available carbohydrate constituents in annual morning-glory. Bot. Gaz. 107:120-129. 1945.

112. Mitchell, J. W., R. H. Burris and A. J. Riker. Inhibition of respiration in plant tissues by callus stimulating substances and related chemicals. Amer. J. Bot. 36:368-377. 1949.

113. Moewus, Franz. The action of 2,4-D on deaminating enzymes. 8th International Bot. Congress. pp. 149-150. 1954.

114. Nance, J. F. Inhibition of salt accumulation in excised wheat roots by 2,4-dichlorophenoxyacetic acid. Science 109:174-176. 1949.

115. Neely, W. B., C. D. Ball, C. L. Hamner and H. M. Sell. Effect of 2,4-dichlorophenoxyacetic acid on the alpha and beta amylase activity in the stems and leaves of red kidney bean plants. Science 111: 118. 1950.

116. Nickell, L. G. Effect of certain plant hormones and colchicine on the growth and respiration of virus tumor tissue from Rumex acetosa. Amer. J. Bot. 37:829-835. 1950.

117. Ordin, L., R. Cleland and J. Bonner. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32:216-220. 1957.

118. Ormrod, D. P. and W. A. Williams. Phosphorus metabolism of Trifolium hirtum All. as affected by 2,4-dichlorophenoxyacetic acid and gibberellic acid. Plant Physiol. 35:81-87. 1960.

119. Payne, M. G., J. L. Fults and R. J. Hay. Amino acids in potato tubers altered by 2,4-D treatment of plants. Science 114:204-205. 1951.

120. Rakitin, Y. V. and A. K. Potapova. Penetration into plants of herbicides and their influence on phosphorus uptake. Fiziologiia Rastenii. 6:621-623. 1960.

121. Rakitin, Y. V. and V. A. Zemskaia. The effect of 2,4-D on nitrogen metabolism in oat and bean plants. Fiziologiia Rastenii. 5:190. 1958.

122. Rasch, E., H. Swift and R. M. Klein. Nucleoprotein changes in plant tumor growth. J. Biophysic. and Biochem. Cytol. 6:11-34. 1959.

123. Rasmussen, L. W. The physiological action of 2,4-dichlorophenoxyacetic acid in dandelion, Taraxacum officinale. Plant Physiol. 22:377-392. 1947.

124. Rebstock, T. L., C. L. Hamner, C. D. Ball and H. M. Sell. Effect of 2,4-dichlorophenoxyacetic acid on proteolytic activity of red kidney bean plants. Plant Physiol. 27:639-643. 1952.

125. Rebstock, T. L., C. L. Hamner and H. M. Sell. The influence of 2,4-dichlorophenoxyacetic acid and the phosphorus metabolism of cranberry bean plants (Phaseolus vulgaris). Plant Physiol. 29:490-491. 1954.

126. Rhodes, A. The influence of the plant growth-regulator, 2-methyl-4-chlorophenoxyacetic acid, on the metabolism of carbohydrate, nitrogen and minerals in Solanum lycopersicum. J. Exp. Bot. 3:129-154. 1952.

127. Rhodes, A., W. G. Templeman and M. N. Thruston. The effect of plant growth regulator, 4-chloro-2-methyl phenoxyacetic acid, on the mineral and nitrogen content of plants. Ann. Bot. (n.s.) 14:181-198. 1950.

128. Rice, E. L. and L. M. Rohrbaugh. Relation of potassium nutrition to the translocation of 2,4-dichlorophenoxyacetic acid in tomato plants. Plant Physiol. 33:300-303. 1958.

129. Roe, J. H. A colorimetric method for the determination of fructose in blood and urine. J. Biol. Chem. 107:15-22. 1934.

130. Rohrbaugh, L. M. and E. L. Rice. Relations of phosphorus nutrition to the translocation of 2,4-dichlorophenoxyacetic acid in tomato plants. Plant Physiol. 31:196-199. 1956.

131. Sell, H. M., C. L. Hamner, T. L. Rebstock and L. E. Weller. The effect of monochlorophenoxyacetic acids on the composition of bean plants (Phaseolus vulgaris). Quart. Bull. of the Mich. Agric. Exp. Sta. 40:306-310. 1957.

132. Sell, H. M., R. W. Luecke, B. M. Taylor and C. L. Hamner. Changes in chemical composition of stems of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 24:295-299. 1949.

133. Shaw, W. C., C. J. Wilard and R. L. Bernard. The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on wheat, oats, barley and the legumes underseeded in these crops. Ohio Agr. Exp. Sta. Res. Bull. 761. 1955.

134. Sibley, J. A. and A. L. Lehninger. Determination of aldolase in animal tissues. J. Biol. Chem. 177:859-872. 1949.

135. Sie, Hsien-Giett, V. N. Nigam and W. H. Fishman. Conversion of nucleoside to sedoheptulose monophosphate by rat liver. J. Biol. Chem. 234:1202-1207. 1959.

136. Silberger, J. and F. Skoog. IAA-induced changes in nucleic acid contents and growth of tobacco pith tissue. Science 118:443-444. 1953.

137. Singer, T. P. Plant Carboxylases. In Methods of Enzymology. I. S. P. Colowick and N. O. Kaplan, Ed. pp. 460-471. New York: Academic Press. 1957.

138. Singer, T. P. and J. Pensky. Isolation and properties of the alpha-carboxylase of wheat germ. J. Biol. Chem. 196:375-388. 1952.

139. Skoog, F. Substances involved in normal growth and differentiation of plants. Brookhaven Symposia in Biology 6(BNL 258):1-21. 1954.

140. Skoog, F. and B. J. Robinson. A direct relationship between indoleacetic acid effects on growth and reducing sugars in tobacco tissue. Proc. Soc. Exp. Biol. Med. 74:565-568. 1950.

141. Slein, M. W. Phosphoglucose Isomerase. In Methods in Enzymology. I. S. P. Colowick and N. O. Kaplan, Ed. pp. 304-306. New York: Academic Press. 1955.

142. Smith, F. G. Effects of 2,4-D on the respiratory metabolism of bean stem. Plant Physiol. 23:70-80. 1948.

143. Smith, F. G., C. L. Hamner and R. F. Carlson. Changes in food reserves and respiratory capacity of bindweed tissues accompanying herbicidal action of 2,4-D. Plant Physiol. 22:58-65. 1947.

144. Smith, I. Ed. Chromatographic Techniques, 1st Ed. New York: Interscience Publishers, Inc. 1958.

145. Stahler, L. M. and E. I. Whitehead. The effect of 2,4-D on potassium nitrate levels in leaves of sugar beets. Science 112:749-751. 1950.

146. Stockell, Anne. The binding of diphosphopyridine nucleotide by glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. 234:1286-1292. 1959.

147. Switzer, C. M. Effects of herbicides and related chemicals on oxidation and phosphorylation by isolated soybean mitochondria. Plant Physiol. 32:42-44. 1957.

148. Tagawa, T. and J. Bonner. Mechanical properties of the Avena coleoptile as related to auxin and to ionic interactions. Plant Physiol. 32:207-215. 1957.

149. Taylor, D. L. Effects of 2,4-D on gas exchange of wheat and mustard seedlings. Bot. Gaz. 109:162-176. 1948.

150. Theorell, H. Über Hemmung der Reaktions-geschwindigkeit durch Phosphat in Warburgs und Christians System. Biochem. Z. 275:416-421. 1935.

151. Tukey, H. B., C. L. Hamner and B. Moore. Histological changes in bindweed and sow thistle following applications of 2,4-D in herbicidal concentrations. Bot. Gaz. 107:62-73. 1945.

152. Umbreit, W. W., R. H. Burris and J. F. Stauffer. Manometric Techniques, 3rd Ed. pp. 28-45. Minneapolis: Burgess. 1957.

153. Velick, S. F. The alcohol and glyceraldehyde-3-phosphate dehydrogenases of yeast and mammals. In Mechanisms of Enzyme Action. W. C. McElroy and B. Glass, Ed. pp. 491-519. Baltimore: Johns Hopkins Press. 1954.

154. Velick, S. F. Flouresence spectra and polarization of glyceraldehyde-3-phosphate and lactic dehydrogenase co-enzyme complexes. J. Biol. Chem. 233:1455-1467. 1958.

155. Vennesland, B. and E. Conn. Carboxylating enzymes in plants. Ann. Rev. Plant Physiol. 3: 307-332. 1952.

156. Volker, J. F. Compounds capable of plant amylase inhibition. Science 112:61. 1950.

157. Wagenknecht, A. C., A. J. Riker, T. C. Allen and R. H. Burrss. Plant growth substances and the activity of cell-free respiratory enzymes. Amer. J. Bot. 38:550-554. 1951.

158. Warburg, O. and W. Christian. Isolierung und Kristallisation des Proteins des oxydierenden Gärunsferments. Biochem. Z. 303:40-68. 1939.

159. Wedding, R. T., L. C. Erickson and B. L. Brannaman. Effect of 2,4-D on photosynthesis and respiration. Plant Physiol. 29:64-69. 1954.

160. Wedding, R. T., L. C. Erickson and M. Kay Black. Influence of 2,4-dichlorophenoxyacetic acid on solute uptake by Chlorella. Plant Physiol. 34: 3-10. 1959.

161. Weller, L. E., R. W. Leuke, C. L. Hamner and H. M. Sell. Changes in chemical composition of the leaves and roots of red kidney beans treated with 2,4-D. Plant Physiol. 25:289-293. 1950.

162. West, F. R., Jr. and J. H. M. Henderson. The effect of 2,4-dichlorophenoxyacetic acid and various other substances upon the respiration of blue lupine seedling roots. Science 111:579-581. 1950.

163. West, S. H., J. B. Hanson and J. L. Key. Effect of 2,4-dichlorophenoxyacetic acid on the nucleic acid and protein content of seedling tissue. Weeds In Press.

164. Wilden, C. E., C. L. Hamner and S. T. Bass. The effect of 2,4-dichlorophenoxyacetic acid on the accumulation of mineral elements in tobacco plants. Plant Physiol. 32:243-244. 1957.

165. Wolf, D. E., G. Vermillion, A. Wallace and G. H. Ahlgren. Effect of 2,4-D on carbohydrate and nutrient element content and on rapidity of kill of soybean plants growing at different nitrogen levels. Bot Gaz. 112:188-197. 1950.

166. Wort, D. J. Effects of non-lethal concentrations of 2,4-D on buckwheat. Plant Physiol. 26:50-58. 1951.

167. Wort, D. J. The response of buckwheat to treatment with 2,4-dichlorophenoxyacetic acid. Amer. J. Bot. 36:673-676. 1949.

168. Wort, D. J. and L. M. Cowie. The effect of 2,4-D on phosphorylase, phosphatase, amylase, catalase and peroxidase activity in wheat. Plant Physiol. 28:135-139. 1953.

169. Wu, R. Regulatory mechanisms in carbohydrate metabolism. V. Limiting factors of glycolysis in HeLa cells. J. Biol. Chem. 234:2806-2810. 1959.

170. Wu, R. and E. Racker. Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumor cells. J. Biol. Chem. 234:1029-1035. 1959.

171. Wu, R. and E. Racker. Regulatory mechanisms in carbohydrate metabolism. IV. Pasteur effect and Crabtree effect in ascites tumor cells. J. Biol. Chem. 234:1036-1041. 1959.

172. Yasuda, G. K., M. G. Payne and J. L. Gults. Effect of 2,4-dichlorophenoxyacetic acid and maleic hydrazide on potato proteins as shown by paper electrophoresis. Nature 176:1029-1030. 1956.

BIOGRAPHICAL SKETCH

Clanton C. Black, Jr., was born November 27, 1931, in Tampa, Hillsborough County, Florida. He attended elementary school at Mango, Florida, until he was ten years old. He completed his education at Gainesville, Florida.

After graduation from Gainesville High School in 1949, he entered the University of Florida, where he majored in Agronomy. During his third year of college, he married Betty L. Dantzler. The Degree of Bachelor of Science in Agriculture was conferred on him by this same institution in August, 1953.

He returned to the University of Florida in September, 1955, to pursue graduate work in the field of Agronomy, specializing in Chemical Weed Control. The Degree of Master of Science in Agriculture was conferred on him in June, 1957.

In September, 1957, he began further graduate studies at the University of Florida, majoring in Agronomy. In August, 1960, the Degree of Doctor of Philosophy was conferred on him.

He is a member of the following honorary societies: Gamma Sigma Delta, Phi Sigma and Sigma Xi.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1960

M. A. Brunson
Dean, College of Agriculture

Dean, Graduate School

Supervisory Committee:

E. J. Rodgers
Chairman

J. E. Humphreys

I. V. Stearns

O. Charles Ruelke

D. D. Smith